MYCORRHIZAL INOCULATION, ENDOPHYTIC COLONIZATION, AND ALLELOPATHIC POTENTIAL OF WOLLEMI PINE (*Wollemia nobilis*) ROOTS

by

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Abstract

Mycorrhizas are ubiquitous symbiotic relationships between soil-inhabiting fungi and the roots of over 90 % of terrestrial plants. Mycorrhizal colonization of *Wollemia nobilis* Jones, Hill & Allen (Wollemi Pine), a newly discovered and extremely rare conifer native to southeastern Australia, is a largely unexplored subject. The objectives of this work were: (1) to assess mycorrhizal colonization of Wollemi Pines following fungal inoculation, (2) to identify endophytic fungi associated with Wollemi Pine roots growing in the field and in horticultural substrates, and (3) to evaluate the allelopathic activity of Wollemi Pines. In both laboratory and field environments, juvenile trees were inoculated with several different arbuscular mycorrhizal fungi and one fungus known to form ectendomycorrhizas on members of the Pinaceae. Mycorrhizal colonization was not evident in any of the inoculated plants; however, endophytic fungal structures formed by *Cylindrocarpon pauciseptatum* and *Phialocephala fortinii* were consistently present in the roots of plants grown in the field or in growth chambers. Preliminary evidence of allelopathic chemical production by Wollemi Pines was also found. Extracts of soil used to grow Wollemi Pines suppressed germination of leek seeds but not sorghum seeds. This thesis presents the results from the first mycorrhizal fungal inoculation of Wollemi Pine, the first identification of fungal endophytes colonizing Wollemi Pine roots, and the first evidence for the production of allelopathic chemicals by Wollemi Pine.

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Dedication

For my family and friends, who offered me enduring support and encouragement throughout the course of this thesis.

Co-Authorship Statement

Dr. Daniel Durall and Dr. Susan Murch assisted in the design and setup of this research, and provided guidance in both data analysis and thesis preparation. I was responsible for setting up and conducting the experiments, as well as analyzing the data and writing the thesis.

Chapter 1: Introduction

1.1 Wollemi Pine: One of the Oldest and Rarest Extant Plant Species

The Wollemi Pine, *Wollemia nobilis* Jones, Hill & Allen, was recently discovered by David Noble in a remote area of Wollemi National Park, about 150 kilometres northwest of Sydney, Australia (Jones et al., 1995). With fewer than 40 mature trees and 200 seedlings known to exist in this area, it is one of the rarest trees in the world (Offord and Meagher, 2006). The trees are located in a moist rainforest environment surrounded by coachwood (*Ceratopetalum apetalum*), sassafras (*Doryphora sassafras*), and a variety of ferns and eucalypts (Bardell, 2006). Mature Wollemi Pines grow up to 40 m tall and some are estimated to be at least 500 years old (Offord and Meagher, 2006). Since their discovery in 1994, information regarding the exact location of the trees has been restricted to prevent exploitation of the remaining populations (Briggs, 2000). Cuttings from wild Wollemi Pines have been propagated to maintain botanic garden specimens used in public education and conservation research (Offord and Meagher, 2006) (Figure 1.1).

Figure 1.1 Young *Wollemia nobilis* trees propagated from cuttings in Australia and used for research at UBC Okanagan in Kelowna, British Columbia.

Taxonomic classification of Wollemi Pine proved initially difficult due to the unique appearance of the specimens that were collected. Chloroplast DNA sequences provided phylogenetic evidence for the taxonomic placement of Wollemi Pine as a distinct monotypic genus, *Wollemia*, in the family Araucariaceae (Gilmore and Hill, 1997). In addition, the chromosome number of Wollemi Pine (2n = 26) is consistent with that of other members of the Araucariaceae (Hanson, 2001). This ancient conifer family has been used in climate reconstruction studies and has an extensively studied fossil record worldwide (Chambers et al., 1998). According to fossil records, members of the Araucariaceae were widespread in the Jurassic and Early Cretaceous periods, from 200 million years ago until 65 million years

ago (Kershaw and Wagstaff, 2001). Coinciding with the event that caused extinction of the dinosaurs, this plant family became greatly reduced in number of species and restricted to the southern hemisphere (Kershaw and Wagstaff, 2001). Morphologically, the broad leaves, wingless pollen, nodular root structure, and large female strobili all unmistakably placed the Wollemi Pine in the Araucariaceae (Jones et al., 1995). There are some features of the Wollemi Pine that are distinct from the two extant genera in the Araucariaceae, *Agathis* and *Araucaria*, thus supporting creation of the new genus (Jones et al., 1995). For example, dark nodular bark forms in spongy layers up to 20 mm thick on mature Wollemi Pines, but is not present in Agathis or Araucaria (Jones et al., 1995). The Wollemi Pine also has features that are shared with one genus or the other. For example, the leaf anatomy of *W. nobilis* is more similar to that of *Araucaria* due to the arrangement of the spongy and palisade mesophyll tissue as well as the presence of stomata on the upper and lower leaf surfaces (Burrows and Bullock, 1999). However, the resins produced by Wollemi Pines are virtually identical to those produced by *Agathis* (Lambert et al., 1999). Also, the chemical composition of the *W. nobilis* leaf essential oil is more similar to that of *Agathis* than *Araucaria* (Brophy et al., 2000).

Experiments involving Wollemi Pine have revealed the fragility of the remaining populations. Laboratory-based inoculations of Wollemi Pines with pathogenic fungi showed that the trees were extremely susceptible to infection, particularly by *Phytophthora cinnamomi* and *Botryosphaeria* sp. (Bullock et al., 2000). A study comparing polymorphisms at three different loci of 26 wild Wollemi pine samples determined that the existing trees have extremely low genetic diversity (Peakall et al., 2003). Collectively, these results suggest that the existing Wollemi Pine stands are extremely vulnerable to introduced

pathogens, thus underlining the importance of strictly regulating access to the remaining trees growing under natural conditions.

In addition to conserving existing Wollemi Pines, future conservation strategies may also include attempts to increase population size via outplanting laboratory-propagated seedlings. Outplanting efforts require a comprehensive understanding of how Wollemi Pines acclimate to their surroundings and interact with nearby organisms. Planting laboratory-propagated trees near the existing population is challenged by the nature of the soil, which is a Hawkesbury Sandstone-derived soil with high organic matter and low pH (Jones et al., 1995). This soil type has been studied for several decades because of its low water-retaining capacity and minimal nutrient content, especially that of phosphorus (P) (Beadle, 1962). Phosphorus levels in Hawkesbury Sandstone-derived soil can be as low as 30 mg/kg and are a major factor in determining the plant communities that can grow on these soils (Beadle, 1962).

The main P source in Hawkesbury Sandstone-derived soil is insoluble organic P, while soluble P sources are in short supply (Beadle, 1962; Jones et al., 1995). While mature Wollemi Pines are found growing in this soil type (Jones et al., 1995), the establishment of outplanted seedlings in this environment may prove difficult. Many Australian native plants are adapted to growing on low-nutrient soils and have developed mechanisms to take up and use nutrients as efficiently as possible (Thomson and Leishman, 2004). For example, Bellgard (1991) examined the roots of numerous native Australian plants growing on nutrient deficient sandstone-derived soils and found that the majority of them were mycorrhizal. Most of the studied plants were associated with arbuscular mycorrhizal fungi (see section 1.2.2.1), while few were associated with ectomycorrhizal fungi (see section 1.2.2.2)

(Bellgard, 1991). Therefore, laboratory-propagated Wollemi Pine seedlings may have difficulty establishing in nutrient-deficient soils without harbouring mycorrhizal fungi in their roots.

McGee et al. (1999) published the only research paper examining mycorrhizal associations formed by Wollemi Pine. They sampled roots from two mature trees growing in the wild and three seedlings growing in the Royal Botanic Gardens in Australia (McGee et al., 1999). The roots of juvenile trees were not colonized, but mature trees were consistently mycorrhizal (McGee et al., 1999). All of the mycorrhizas found were *Paris*-type arbuscular mycorrhizas (see section 1.2.2.1), with the exception of one adult tree with ectendomycorrhizas in one root sample (see section 1.2.2.3) (McGee et al., 1999). Nevertheless, it is difficult to reach any conclusion from this study due to the small number of samples that were examined. Future research directions include verifying that Wollemi Pines form mycorrhizal associations, identifying mycorrhizal fungal species compatible with Wollemi Pine, and evaluating the effects of mycorrhizas on nutrient uptake by trees growing in sandstone-derived soil.

1.2 The Importance of Mycorrhizas in Plant Fitness

1.2.1 Prevalence

Colonization of terrestrial vascular plant roots by mycorrhizal fungi is exceedingly common (Newman and Reddell, 1987). Some of the highly conserved structures formed by some types of mycorrhizas are present in fossils estimated to be approximately 400 million years old (Peterson et al., 2004), indicating that mycorrhizas are adaptable and evolved

symbioses that have persisted throughout numerous climatic and geographic challenges. Fossil dating and DNA analyses have confirmed that arbuscular mycorrhizal fungi (described in section 1.2.2.1) diverged from other fungal groups between 353 and 462 million years ago (Simon et al., 1993; Redecker et al., 2000). This time period coincides with the colonization of land by ancient aquatic plants, thus supporting the hypothesis that arbuscular mycorrhizal fungi were instrumental in the early success of terrestrial plants (Simon et al., 1993). Currently, mycorrhizas are formed by 90 % of vascular plants that have been studied, with the exceptions being most members of the Caryophyllaceae, Brassicaceae, Chenopodiaceae, Cyperaceae, and Juncaceae (Newman and Reddell, 1987; Bolan, 1991; Fitter and Moyersoen, 1996). Vascular plants that grow independently of mycorrhizal associations tend to be ruderal species occupying disturbed areas or aquatic species in habitats lacking sufficient oxygen for mycorrhizal fungal respiration (Mejstrik, 1972). Fungi that are capable of forming mycorrhizas include thousands of members of the Phyla Glomeromycota, Ascomycota, and Basidiomycota and are found worldwide in a variety of habitats (Smith and Read, 1997). The involvement of such a large number of plant and fungal symbionts in a diverse range of habitats indicates that mycorrhizas are one of the most ecologically important and widespread symbioses on earth.

1.2.2 Types of Mycorrhizas

Mycorrhizas are currently divided into several distinctive groups based on the taxonomy of the symbionts and the anatomy of the symbiotic organ formed (Peterson et al., 2004). Most research has focused on ectomycorrhizas (section 1.2.2.2), characterized by the formation of a mantle and intercellular Hartig net, and arbuscular mycorrhizas (section

1.2.2.1), characterized by intracellular hyphal complexes called arbuscules. Mainly the latter have been detected in mature Wollemi Pine roots; however, structures resembling ectendomycorrhizas (section 1.2.2.3) have also been observed on one occasion in mature Wollemi Pine roots (McGee et al., 1999). Ectendomycorrhizas, which have received little attention, are considered to be a subset of ectomycorrhizas because of the development of both a mantle and intracellular hyphae.

1.2.2.1 Arbuscular mycorrhizas

Arbuscular mycorrhizas are the most common mycorrhizal type, having been found in over 80 % of terrestrial vascular and several non-vascular plant species (Peat and Fitter, 1993). The fungi that form arbuscular mycorrhizas belong to the Phylum Glomeromycota, which consists of eight genera and approximately 150 species (Schüβler et al., 2001). These fungi are normally present within colonized roots or as hyphae or spores, which germinate under optimal conditions and produce germ tubes (Tawaraya et al., 2007). Hyphal contact with a root hair or epidermal cell results in the formation of an attachment structure called an appressorium, from which the internal fungal structures will develop (Peterson et al., 2004; Tawaraya et al., 2007). At this stage there are two types of arbuscular mycorrhizas that may form based on the colonization patterns of the mycobiont. *Arum*-type arbuscular mycorrhizas are characterized by extensive intercellular hyphae in the root cortex, as well as simple intracellular hyphal coils and tree-like arbuscules formed by the repeated branching of a single intracellular trunk hypha (Dickson, 2004; Peterson et al., 2004). In contrast, *Paris*-type mycorrhizas develop numerous complex intracellular coils in root cortical cells, off of which small arbuscules form by repeated lateral branching (Dickson, 2004; Peterson et al.,

2004). Despite the range of angiosperms, gymnosperms, pteridophytes, and bryophytes that form arbuscular mycorrhizas, research on this symbiosis has tended to focus on colonization of crop plants such as wheat, rice, corn, and cotton (Peterson et al., 2004). Such studies often concentrate on the potential to improve the health of some crop plants by adding arbuscular mycorrhizal fungal propagules to large-scale agricultural operations (e.g. Sutton, 1973; Farmer et al., 2007). The *Arum*-type, found mostly in cultivated herbs and crop plants, is less common than the *Paris*-type, which form predominately in trees, forest herbs, and wild angiosperms (Dickson, 2004).

In addition to arbuscules and intraradical hyphae, both types of arbuscular mycorrhizas often form structures such as intraradical vesicles, extraradical auxiliary vesicles, asexual spores, and extraradical mycelium (Peterson et al., 2004). These structures aid in the detection and identification of arbuscular mycorrhizas, and are important factors in fungal propagation and the uptake and storage of soil nutrients (Peterson et al., 2004). Also, the spores and extraradical hyphae secrete a glycoprotein called glomalin (Wright and Upadhyaya, 1996), which has been shown to influence soil aggregation and improve soil structure (Rillig et al., 2002).

1.2.2.2 Ectomycorrhizas

Ectomycorrhizal associations are not as widespread as arbuscular mycorrhizas, but tend to dominate in forest ecosystems. Ectomycorrhizas have been detected in three percent of studied gymnosperm and angiosperm tree species as well as a small number of shrubs and herbs (Smith and Read, 1997). Although this mycorrhizal type has not been previously identified in Wollemi Pine or other members of the Araucariaceae (McGee et al., 1999),

more research is required to completely ascertain that this association is absent. The role of ectomycorrhizas in forest ecology and management has been widely studied, particularly in logged and disturbed sites (e.g. Molina, 1982; Jones et al., 2003). The 6000 fungal species capable of forming ectomycorrhizas belong to the Phyla Basidiomycota, Ascomycota, and Zygomycota (Peterson et al., 2004).

Formation of the ectomycorrhizal association begins with a fungal sheath encasing the roots (Smith and Read, 1997). The sheath, also known as a mantle, is a diverse structure composed of compact hyphae of varying colours, textures, and thicknesses (see Agerer, 1991). The hyphae of the inner mantle branch repeatedly to form the Hartig net, an intercellular network of hyphal cells in the root cortex (Blasius et al., 1986). Extraradical mycelium develops when hyphae from the outer mantle extend away from the root, forming numerous connections between the mantle and the soil (Peterson et al., 2004). Hyphal connections between nearby plants may also occur, and have been shown to facilitate interplant nutrient transfer in both laboratory and field experiments (Simard et al., 1997; McKendrick et al., 2000). In some cases, the extraradical mycelium develops into linear hyphal aggregations called rhizomorphs or compact mycelial masses called sclerotia (Duddridge et al., 1980; Piché and Fortin, 1982). Some ectomycorrhizas may also form tubercules, which are root clusters of ectomycorrhizas contained within a tough outer peridium composed of compact hyphal layers (Peterson et al., 2004). Also, ectomycorrhizal fungi form epigeous or hypogeous sporocarps, which are useful in detecting and identifying the fungi and are often an important food source for small mammals (Johnson, 1996).

1.2.2.3 Ectendomycorrhizas

Ectendomycorrhizas represent relatively uncommon associations that tend to develop in nurseries and disturbed sites on a limited number of tree species (Mikola, 1965; Yu et al., 2001). The fungi that form this mycorrhizal type have been detected in a variety of hosts in forest communities (Tedersoo et al., 2006), and are capable of forming ectendomycorrhizas and ectomycorrhizas in *Pinus*, *Larix*, *Picea*, and *Betula* spp. (Massicotte et al., 1999; Yu et al., 2001). However, the ectendomycorrhizal morphology appears to be limited to *Pinus* and *Larix* hosts (Yu et al., 2001), with one occurrence also reported on mature Wollemi Pine roots (McGee et al., 1999). Fungi forming ectendomycorrhizas were once collectively grouped as E-strain fungi because a lack of detectable sexual stages prevented definitive taxonomic classification (Mikola, 1965). Several years later, the ascomycete *Tricharina* was identified as an ectendomycorrhizal mycobiont and was later named *T. mikolae* by Yang and Wilcox (1984). The genus *Tricharina* was then separated into *Tricharina* and *Wilcoxina* based on differences in both ascospore structure and ecological niches, and this separation has since been verified by DNA sequence analysis (Yang and Korf, 1985; Egger, 1996). The main ecological difference between the two genera is that *Tricharina* species tend to be saprobic, while *Wilcoxina* species form mycorrhizal associations (Yang and Korf, 1985). Following the creation of the *Wilcoxina* taxon, two ectendomycorrhizal species were named: *W. rehmii* and *W. mikolae* (Egger et al., 1991).

Ectendomycorrhizal colonization by *Wilcoxina* spp. is characterized by the presence of a thin sheath-like mantle external to the root and a Hartig net, in conjunction with intracellular hyphal development in the root epidermis and cortex (Scales and Peterson, 1991; Yu et al., 2001). Typical ectendomycorrhizal morphology tends to be restricted to

seedlings (Yu et al., 2001), but some studies have shown that this is not always the case (Harley and Harley, 1987; Danielson and Pruden, 1989; McGee et al., 1999). The ectomycorrhizas formed by *Wilcoxina* spp. are similar, but lack the intracellular hyphae.

The wide distribution and host range of *Wilcoxina* spp. indicates a potentially significant role in the growth and survival of host plant species. Most research on the role of *Wilcoxina* colonization in plant health has focused on plants establishing in disturbed or nutrient-deficient areas (Ursic et al., 1997; Yu et al., 2001). For example, studies have shown that *W. mikolae* is capable of surviving in saline and alkaline habitats that do not support the growth of most other fungi (Kernaghan et al., 2002). In addition, seedlings of jack pine that were grown under salt stress exhibited reduced tissue sodium concentrations when colonized with *W. mikolae* as compared to uncolonized plants (Calvo Polanco et al., 2008). Prabhu et al. (1996) reports ferricrocin formation by *Wilcoxina* spp., although it is not known if the siderophores are used to solubilize iron for plant uptake or as a mechanism to compete with the host for iron. *Wilcoxina* spp. are also capable of producing acid phosphatases, which help solubilize phosphorus from forms that may be otherwise unavailable to host plants (Redlak et al., 2001). A recent study by Jones et al. (2009) found that *Picea engelmannii* seedlings naturally colonized by a *Wilcoxina* sp. accumulated more ^{15}N than seedlings colonized by other ectomycorrhizal fungi such as *Cenococcum* sp. and *Amphinema* sp. In addition, seedlings of balsam poplar inoculated with *W. mikolae* showed increased shoot growth and root volumes as compared to uninoculated control plants (Siemens and Zwiazek, 2008). Although the available literature suggests that *Wilcoxina* spp. are capable of surviving in severe conditions and solubilizing some mineral nutrients, more research is required to link these activities to improved plant health under natural conditions.

1.2.3 Effects of Mycorrhizas

Mycorrhizas are often described as mutualisms, in which both symbiotic partners receive some benefit from the association (Smith and Read, 1997). However, increasing evidence of exceptions to this definition has prompted a re-examination of the symbiotic classification of mycorrhizas (Johnson et al., 1997). Depending on the host:fungus combination, some mycorrhizal associations are considered closer to parasitic on the plant partner than mutualistic, but are still considered mycorrhizas (see Smith and Read, 1997). Other mycorrhizal types, such as ectendomycorrhizas, have had their functions studied only minimally and the nature of the symbiosis is not totally clear (Peterson et al., 2004). A review by Jones and Smith (2004) argues that the criteria to classify a symbiosis should be based strictly on structural and developmental aspects as opposed to the benefits attributed to each partner. Therefore, this definition includes symbiotic associations between fungi and plants that lie between mutualistism and parasitism but still form structures typical of mutualistic mycorrhizas (Jones and Smith, 2004).

In a mycorrhizal association, the fungal partner benefits by obtaining up to 20 % of the host's net photosynthate (Smith and Read, 1997). The plant may receive several benefits, including pathogen resistance (Newsham et al., 1995; Azcón-Aguilar and Barea, 1996), improved water uptake (Allen and Boosalis, 1983; Levy and Syvertsen, 1983), detoxification of soils contaminated with heavy metals such as cadmium and lead (Gaur and Adholeya, 2004), and increased nutrient uptake (e.g. Joner and Jakobsen, 1995). The most widely studied mycorrhiza-derived benefit to plants is the improved acquisition of mineral nutrients, particularly that of phosphorus (P) and nitrogen (N). Although some nutrients appear to be abundant in soil, many forms of these nutrients are unavailable to plants (Smith and Read,

1997). For example, soil P is often taken up by plants as orthophosphate, but also exists as tightly bound crystal lattice forms or organic forms, such as phospholipids and nucleic acids (Smith and Read, 1997). Orthophosphate is often present at low concentrations and tends to be unevenly distributed in soils (Robinson, 1994). Also, the low mobility of many soil nutrients does not allow them to diffuse to plant roots as rapidly as they are taken up by the plant (Lewis and Quirk, 1967). In response, plants can modify their root structure by exhibiting increased root proliferation, root length, or root hairs in order to have greater access to soil nutrients (Lambers et al., 2006), but all of these modifications require energy. Low nutrient mobility and availability in soil often create a competitive environment favouring plants that are able to access additional nutrient sources.

Mycorrhizal plants often have increased nutrient uptake for several reasons. First, many mycorrhizal fungi are capable of solubilizing forms of nutrients that are unavailable to plants and converting them into usable forms (Smith and Read, 1997). For example, some ectomycorrhizal fungi secrete an iron and aluminum chelating agent called calcium oxalate, which releases bound nutrients such as P for plant uptake (Malajczuk and Cromack, 1982). In addition, some ectomycorrhizal and arbuscular mycorrhizal fungi are capable of solubilizing nutrients through the production and release of enzymes, such as phosphateas (Joner and Johansen, 2000; Chen et al., 2001). Also, the extramatrical hyphae of mycorrhizal fungi are often finer and longer than root hairs, allowing greater physical exploration of the soil volume well beyond the depletion zone (Sanders and Tinker, 1973). Extramatrical hyphal development also provides a greater surface area for nutrient absorption (Bolan, 1991). Most of the work on the role of hyphae in nutrient uptake has historically focused on arbuscular mycorrhizas (e.g. Stribley et al., 1980). However, Jones et al. (1990)

studied ectomycorrhizas in *Salix viminalis* and demonstrated that P uptake was proportional to the length of the extramatrical hyphae of the ectomycorrhizal fungus, being one of the first studies to directly demonstrate the importance of ectomycorrhizas in plant P nutrition. Producing extramatrical hyphae uses less carbon than producing roots of the same length, and nutrients are translocated more efficiently in hyphae than in roots (Smith and Read, 1997), thus providing an economical alternative to extensive root modifications.

In addition to the features described above, mycorrhizal fungi may also improve P uptake by forming inorganic polyphosphate granules in hyphal vacuoles. Polyphosphates are rapidly synthesized linear polymers of three to thousands of phosphate residues that are used to store P for release during times of P stress (Ohtomo and Saito, 2005). As fungal hyphae take up inorganic P from the soil and convert it into polyphosphate granules, the internal concentration of phosphate is lowered in the hyphae, thus maintaining a gradient so that P uptake is not slowed by fungal accumulation (Rasmussen et al., 2000). There have been studies that challenge the evidence for polyphosphate granules, particularly in ectomycorrhizas, as some researchers believe they are merely an artefact of specimen preparation (Ashford et al., 1975; Orlovich and Ashford, 1993). Nevertheless, Takanishi et al. (2009) recently used enzymatic assays to detect polyphosphate granules in arbuscular mycorrhizal roots of *Allium fistulosum*, and found that polyphosphates of less than 20 inorganic P residues were positively correlated with plant growth. Further research is needed to investigate polyphosphate formation as a mechanism used by mycorrhizal fungi to create a reservoir of P for the host plant.

1.2.4 Mycorrhizal Inoculation Techniques

Manipulating mycorrhizal colonization of plants allows researchers to evaluate plant responses to the symbiosis. Techniques for inoculating plants with mycorrhizal fungi have been developed over time and there are several widely accepted inoculation protocols currently in practice (e.g. Abbott and Robson, 1984; Dodd and Thomson, 1994). Inoculation methods are all designed to ensure that viable inoculum contacts the roots as they grow, but there are variations depending on the type of mycorrhizal association and the objectives of the research study (Menge and Timmer, 1982; Riffle and Maronek, 1982; Howeler and Sieverding, 1983). Procedures for inoculum preparation and application may also differ based on the type of inoculum used in the study. Spore or pure inoculum consists only of spores of the fungal symbiont, and is often used because its purity can be confirmed and the spore numbers are quantifiable (Menge and Timmer, 1982). Whole or mixed inoculum, composed of spores, mycelium, and colonized roots, is more commonly used because it is readily obtainable and often has a greater inoculum potential than spores alone (Menge and Timmer, 1982).

1.2.4.1 Ectomycorrhizal Fungal Inoculum

Ectomycorrhizal fungal inoculum can be purchased from a culture collection or isolated directly from the surface of mycorrhizal roots. Ectomycorrhizal fungi can also be isolated from hypogeous or epigeous sporocarp tissue, but the success of this technique varies greatly between fungal species (Molina and Palmer, 1982). Crushing and sieving the sporocarp material is a commonly used method to isolate spores for use as inoculum (Chen et

al., 2006). Once the fungal material has been isolated, the spores that have been recovered may then be quantified and used as spore inoculum (Ferguson and Woodhead, 1982). To develop a local source of inoculum, the fungal material must be propagated using culture media. Most ectomycorrhizal fungi grow readily on nutrient agar such as modified Melin-Norkrans (MMN) or potato dextrose agar (PDA), and can be stored long-term if transferred to fresh media every three to four months (Marx, 1969; Molina and Palmer, 1982). If using a liquid culture medium, a layer of broken glass along the bottom of the culture flask fragments actively growing mycelium and further increases fungal growth (Molina and Palmer, 1982). To prepare the fungus for use as inoculum, the hyphae are removed from culture, vacuum-filtered, rinsed with sterile water, and fragmented in a waring blender (Marx and Kenney, 1982).

Ectomycorrhizal inoculum is often supplied to seeds or seedlings as slurries of mycelium or crushed spores mixed with water, spawn (hyphae grown in sterile peat or peat/vermiculite), agar plugs cut from cultures growing on solid media, or by placing the roots between a sandwich of filter papers colonized by the fungus (Molina and Palmer, 1982; Riffle and Maronek, 1982; Chilvers et al., 1986; Gagné et al., 2006). The paper-sandwich technique is an aseptic inoculation method that involves applying paper-grown inoculum to seedling roots on an agar plate of mineral salts medium (Chilvers et al., 1986). This method is widely used because it provides rapid colonization and ensures direct contact of inoculum and roots under axenic conditions (Chilvers et al., 1986). Large-scale field or lab experiments often require broadcast inoculation, in which the inoculum is evenly mixed with the substrate in pots or at planting sites (Riffle and Maronek, 1982). Also, spores can be

suspended in water and applied using irrigation systems so that they leach into the soil (Marx and Kenney, 1982).

There are recommendations available regarding optimal inoculum densities, but modifications are required depending on the nature of the experiment and type of inoculum. Typical densities include about 2000 spores per 1 L planting pot, or a 1:13 ratio of inoculum to substrate for hyphal inoculum (Daniels and Skipper, 1982). Inoculations in pure culture may incorporate these numbers as guidelines, but the amount of inoculum used will ultimately vary based on the experiment (Molina and Palmer, 1982). Depending on the symbionts and experimental conditions, ectomycorrhizal colonization may be detected as early as two weeks into the experiment (Riffle and Maronek, 1982).

1.2.4.2 Arbuscular Mycorrhizal Fungal Inoculum

The inability of arbuscular mycorrhizal fungi to grow in culture makes inoculum preparation and application techniques different than those for ectomycorrhizal fungi (Williams, 1992). Fungal propagules are commonly retrieved from soil using wet sieving and decanting (Gerdemann and Nicolson, 1963), but other separation techniques include direct observation, gelatin columns, and sucrose gradient centrifugation (Daniels and Skipper, 1982). Selection of the appropriate separation method depends on the propagule density in the soil, the size of the soil sample, and the soil texture (Dodd and Thomson, 1994). Fungal propagules may also be purchased from a culture collection such as the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM). As with ectomycorrhizal fungi, propagation of the fungal material is often required to obtain sufficient inoculum for experimentation. The most common way to

propagate arbuscular mycorrhizal fungi is through the establishment of pot cultures using rapidly colonized trap plants with fibrous root systems, such as leeks and grasses (Snellgrove et al., 1982; Quilambo et al., 2005; Cáceres and Cuenca, 2006). The inoculum is then recovered from the soil as spores and mycelium, or the roots of the trap plants may be chopped into small pieces and used as a component of whole inoculum (Ferguson and Woodhead, 1982).

Arbuscular mycorrhizal inoculum is most often applied using the layering or banding method, which involves forming layers of inoculum alternating with soil below a seed or seedling to encourage root contact with the fungal material (Menge and Timmer, 1982; Martín et al., 2006). As with ectomycorrhizal inoculum, broadcast inoculation may be suitable for large-scale field experiments (Menge and Timmer, 1982). There are commercial growth substrates, such as Premier PRO-MIX® BX (Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada), that contain arbuscular mycorrhizal fungal propagules that form associations with a wide variety of plant species growing in pots or in the field. Although commercial growth substrates containing inoculum are easy to use, the inoculum densities are difficult to quantify and are often lower than recommended by the literature.

Recommended inoculum densities of arbuscular mycorrhizal fungi vary depending on the research study and inoculum type. A 1:10 to 1:15 ratio of inoculum to substrate is commonly used for whole inoculum, but for studies using spore inoculum, 2000 spores per litre of substrate is suggested (Ferguson and Woodhead, 1982). Typically, arbuscular mycorrhizal colonization between compatible symbionts should develop within eight to ten weeks in laboratory experiments (Ning and Cumming, 2001). Field studies introduce

additional factors, such as competitive interactions and climate changes, which may produce variations in colonization time.

1.2.4.3 Evaluating Root Colonization by Mycorrhizal Fungi

Inoculation studies are often designed to measure the effects of colonization on the plant, but it is essential to first verify that colonization occurred. Microscope-based techniques often provide a reasonable account of colonization, but molecular identification tools are also used to isolate fungal DNA that may be present on or within the roots. Ectomycorrhizal mantle structure is commonly observed under a dissecting microscope and is an important part in morphotype identification of the fungal symbiont (Agerer, 1991). The definitive characteristic, however, is the presence of a Hartig net, which is often only visible under a compound microscope. Internal root structures formed by arbuscular mycorrhizas are revealed using light microscopy. It is common to treat roots with hot potassium hydroxide and stain the remaining fungal structures with non-vital stains such as trypan blue (Phillips and Hayman, 1970) or chlorazol black E (Brundrett et al., 1984). These stains are frequently used because they permit high resolution of fungal structures within roots (Gange et al., 1999). Arbuscular mycorrhizal colonization of roots can be quantified using either the grid-line intersect method (Giovannetti and Mosse, 1980) or the magnified intersections method (McGonigle et al., 1990). The gridline intersect method involves laying the roots on a 1.25 cm square grid and noting the presence or absence of fungal structures wherever a root intersects a vertical or horizontal gridline. The magnified intersections method requires a light microscope with ocular crosshairs to make several vertical passes of a microscope slide of colonized roots. Each time the ocular crosshairs intersect with a root, an observation is

made regarding the presence or absence of fungal structures. In both methods, the number of intersection points exhibiting colonization is divided by the total number of intersection points to provide a percent colonization value.

1.3 Evidence of Mycorrhizas in the Araucariaceae

There are relatively few publications describing mycorrhizal associations of the Araucariaceae. As described earlier, Wollemi Pines have showed potential to associate with arbuscular mycorrhizal fungi and *Wilcoxina*-like fungi in the wild (McGee et al., 1999). In the same study by McGee et al. (1999), it was found that smooth-barked Kauri Pine (*Agathis robusta*) and Moreton Bay Pine (*Araucaria cunninghamii*) formed only *Paris*-type mycorrhizas. An early research study on the mycorrhizas of Kauri Pine (*Agathis australis*) used soil from mature populations to colonize seedlings with arbuscular mycorrhizal fungi (Morrison and English, 1967). In the same study, it was found that significantly more phosphorus was taken up by colonized Kauri Pine roots than by non-mycorrhizal roots (Morrison and English, 1967). A more recent experiment involving the inoculation of Brazilian Pines (*Araucaria angustifolia*) with the arbuscular mycorrhizal fungus *Glomus clarum* found that the plants were readily colonized by the fungus (Zandavalli et al., 2004). Colonized Brazilian Pines exhibited increased growth responses and tissue macronutrient concentrations as compared to those not exposed to the fungus (Zandavalli et al., 2004). Additional studies examining Brazilian Pines in southern Brazil tropical forests found that the roots were colonized by multiple arbuscular mycorrhizal fungal species, including *Glomus macrocarpum*, *G. etunicatum*, and *G. geosporum* (Breuninger et al., 2000; Moreira-

Souza et al., 2003). A survey of four species from the Podocarpaceae, an ancient conifer family closely related to the Araucariaceae, showed that arbuscular mycorrhizal colonization was common in roots from all of the sampled varieties (Russell et al., 2002). The colonization was detected visually and verified by molecular identification techniques, which revealed multiple fungal symbionts belonging to the Glomeromycota (Russell et al., 2002).

Therefore, based on available scientific literature, plants closely related to Wollemi Pine form arbuscular mycorrhizas but colonization by ectomycorrhizal fungi has not been detected. The ability of the Wollemi Pine to form two types of mycorrhizas makes it unique in its family. More research is required to determine the fungal species involved in the associations and their role in the nutrient uptake of Wollemi Pines.

1.4 Objectives

The overall objective of this work is to determine if Wollemi Pine roots could form ectendomycorrhizas and arbuscular mycorrhizas under laboratory and field conditions in North America. The specific objectives of the study are:

(1) To inoculate Wollemi Pine seedlings with arbuscular mycorrhizal fungi or *Wilcoxina mikolae* and to quantify any colonization.

(2) To measure the effects of inoculation with arbuscular mycorrhizal fungi or *Wilcoxina mikolae* on Wollemi Pine growth and nutrient composition.

(3) To identify fungal endophytes associated with Wollemi Pine roots.

(4) To quantify the effects of extracts of soil in which Wollemi Pine had been growing on the germination of leek and sorghum seeds.

1.5 Overview of Thesis

To achieve the first two objectives listed above, juvenile Wollemi Pines were inoculated using commercial and pot culture inoculum in both laboratory and field experiments. Chapter 2 describes two laboratory-based inoculation experiments using Wollemi Pines that incorporate both *Wilcoxina mikolae* and arbuscular mycorrhizal fungi in different forms of inoculum. Chapter 3 involves a field study integrating local and supplemented arbuscular mycorrhizal fungal inoculum sources. In both chapters, root colonization, plant growth, and plant nutrient composition were quantifed and compared between treatments and to uninoculated trees. Chapter 3 also addresses the third objective by using DNA sequencing methods to identify endophytic fungi in Wollemi Pine roots. The fourth objective is the basis of Chapter 4, which describes an experiment testing the effects of allelochemicals in Wollemi Pine soil extracts on the germination of leek and sorghum seeds. The thesis ends with a concluding chapter containing summarizing remarks about the experiments and recommendations for future work in the application of mycorrhizal fungi to Wollemi Pine conservation.

1.6 References

Abbott LK; Robson AD. (1984) The effect of root density, inoculum placement and infectivity of inoculum on the development of vesicular-arbuscular mycorrhizas. New Phytologist. 97: 285-299

Agerer R. (1991) Characterization of ectomycorrhiza. Methods in Microbiology. 23: 25-73

Allen MF; Boosalis MG. (1983) Effects of two species of VA mycorrhizal fungi on drought tolerance of winter wheat. New Phytologist. 93: 67-76

Ashford AE; Ling Lee M; Chilvers GA. (1975) Polyphosphate in Eucalypt mycorrhizas: a cytochemical demonstration. New Phytologist. 74: 447-453

Azcón-Aguilar C; Barea JM. (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens – an overview of the mechanisms involved. Mycorrhiza. 6: 457-464

Bardell D. (2006) The discovery of a tree (*Wollemia nobilis*) from the age of the dinosaurs. Bios. 77: 20-23

Beadle NCW. (1962) Soil phosphate and the delimitation of plant communities in eastern Australia II. Ecology. 43: 281-288

Bellgard SE. (1991) Mycorrhizal associations of plant species in Hawkesbury Sandstone vegetation. Aust. J. Bot. 39: 357-364

Blasius D; Feil W; Kottke I; Oberwinkler F. (1986) Hartig net structure and formation in fully ensheathed ectomycorrhizas. Nordic Journal of Botany. 6: 837-842

Bolan NS. (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant and Soil. 134: 189-207

Breuninger M; Einig W; Magel E; Cardoso E; Hampp R. (2000) Mycorrhiza of Brazil Pine (*Araucaria angustifolia* [Bert. O. Ktze.]). Plant Biology. 2: 4-10

Briggs BG. (2000) What is significant – the Wollemi Pine or the Southern Rushes? Ann. Missouri. Bot. Gard. 87: 72-80

Brophy JJ; Goldsack RJ; Wu MZ; Fokes CJR; Forster PI. (2000) The steam volatile oil of *Wollemia nobilis* and its comparison with other members of the Araucariaceae (*Agathis* and *Araucaria*). Biochemical Systematics and Ecology. 28: 563-578

Brundrett MC; Piché Y; Peterson RL. (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Canadian Journal of Botany. 62: 2128-2134

Bullock S; Summerell BA; Gunn LV. (2000) Pathogens of the Wollemi pine, *Wollemia nobilis*. Australasian Plant Pathology. 29: 211-214

Burrows GE; Bullock S. (1999) Leaf anatomy of Wollemi pine (*Wollemia nobilis*, Araucariaceae). Aust. J. Bot. 47: 795-806

Cáceres A; Cuenca G. (2006) Contrasting response of seedlings of two tropical species *Clusia minor* and *Clusia multiflora* to mycorrhizal inoculation in two soils with different pH. Trees. 20: 593-600

Calvo Polanco M; Zwiazek JJ; Jones MD; MacKinnon MD. (2008) Responses of mycorrhizal jack pine (*Pinus banksiana*) seedlings to NaCl and boron. Trees. 22: 825-834

Chambers TC; Drinnan AN; McLoughlin S. (1998) Some morphological features of Wollemi Pine (*Wollemia nobilis*: Araucariaceae) and their comparison to cretaceous plant fossils. Int. J. Plant. Sci. 159: 160-171

Chen DM; Taylor AFS; Burke RM; Cairney JWG. (2001) Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. New Phytologist. 152: 151-158

Chen YL; Kang LH; Dell B. (2006) Inoculation of *Eucalyptus urophylla* with spores of *Scleroderma* in a nursery in south China: comparison of field soil and potting mix. Forest Ecology and Management. 222: 439-449

Chilvers GA; Douglass PA; Lapeyrie FF. (1986) A paper-sandwich technique for rapid synthesis of ectomycorrhizas. New Phytologist. 103: 397-402

Daniels BA; Skipper HD. (1982) Methods for the recovery and quantitative estimation of propagules from soil. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 29-35

Danielson RM; Pruden M. (1989) The ectomycorrhizal status of urban spruce. Mycologia. 81: 335-341

Dickson S. (2004) The *Arum-Paris* continuum of mycorrhizal symbioses. New Phytologist. 163: 187-200

Dodd JC; Thomson BD. (1994) The screening and selection of inoculant arbuscular-mycorrhizal and ectomycorrhizal fungi. Plant and Soil. 159: 149-158

Duddridge JA; Malibari A; Read DJ. (1980) Structure and function of mycorrhizal rhizomorphs with special reference to their role in water transport. Nature. 287: 834-836

Egger KN; Danielson RM; Fortin JA. (1991) Taxonomy and population structure of E-strain fungi inferred from ribosomal and mitochondrial DNA polymorphisms. Mycological Research. 95: 866-872

Egger KN. (1996) Molecular systematics of E-strain mycorrhizal fungi: *Wilcoxina* and its relationship to *Tricharina* (Pezizales). Can. J. Bot. 74: 773-779

Farmer MJ; Li X; Feng G; Zhao B; Chatagnier O; Gianinazzi S; Gianinazzi-Pearson V; van Tuinen D. (2007) Molecular monitoring of field-inoculated AMF to evaluate persistence in sweet potato crops in China. Applied Soil Ecology. 35: 599-609

Ferguson JJ; Woodhead SH. (1982) Production of endomycorrhizal inoculum. A. Increase and maintenance of vesicular-arbuscular mycorrhizal fungi. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 47-53

Fitter AH; Moyersoen B. (1996) Evolutionary trends in root-microbe symbioses. Philosophical Transactions: Biological Sciences. 351: 1367-1375

Gagné A; Jany J; Bousquet J; Khasa DP. (2006) Ectomycorrhizal fungal communities of nursery-inoculated seedlings outplanted on clear-cut sites in northern Alberta. Canadian Journal of Forest Research. 36: 1684-1694

Gange AC; Bower E; Stagg PG; Aplin DM; Gillam AE; Bracken M. (1999) A comparison of visualization techniques for recording arbuscular mycorrhizal colonization. New Phytologist. 142: 123-132

Gaur A; Adholeya A. (2004) Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soil. Current Science. 86: 528-534

Gerdemann JW; Nicolson TH. (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society. 46: 235-244

Gilmore S; Hill KD. (1997) Relationships of the Wollemi Pine (*Wollemia nobilis*) and a molecular phylogeny of the Araucariaceae. Telopea. 7: 275-291

Giovannetti M; Mosse B. (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytologist. 84: 489-500

Hanson L. (2001) Chromosome number, karyotype and DNA C-value of the Wollemi pine (*Wollemia nobilis*, Araucariaceae). Botanical Journal of the Linnean Society. 135: 271-274

Harley JL; Harley EL. (1987) A check-list of mycorrhiza in the British flora. New Phytologist (suppl.). 105: 1-102

Howeler RH; Sieverding E. (1983) Potentials and limitations of mycorrhizal inoculation illustrated by experiments with field-grown cassava. Plant and Soil. 75: 245-261

Johnson CN. (1996) Interactions between mammals and ectomycorrhizal fungi. Trends in Ecology and Evolution. 11: 503-507

Johnson NC; Graham JH; Smith FA. (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytologist. 135: 575-585

Joner EJ; Jakobsen I. (1995) Uptake of ^{32}P from labelled organic matter by mycorrhizal and non-mycorrhizal subterranean clover (*Trifolium subterraneum* L.). Plant and Soil. 172: 221-227

Joner EJ; Johansen A. (2000) Phosphatase activity of external hyphae of two arbuscular mycorrhizal fungi. Mycological Research. 104: 81-86

Jones MD; Durall DM; Cairney JWG. (2003) Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging. New Phytologist. 157: 399-422

Jones MD; Durall DM; Tinker PB. (1990) Phosphorus relationships and production of extramatrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. New Phytologist. 115: 259-267

Jones MD; Grenon F; Peat H; Fitzgerald M; Holt L; Philip LJ; Bradley R. (2009) Differences in ^{15}N uptake amongst spruce seedlings colonized by three pioneer ectomycorrhizal fungi in the field. Fungal Ecology. 2: 110-120

Jones WG, Hill KD, Allen JM. (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. Telopea. 6: 173-176

Jones MD; Smith SE. (2004) Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Can. J. Bot. 82: 1089-1109

Kernaghan G; Hambling B; Fung M; Khasa D. (2002) In vitro selection of boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. Restoration Ecology. 10: 43-51

Kershaw P; Wagstaff B. (2001) The southern conifer family Araucariaceae: history, status and value for paleoenvironmental reconstruction. Annu. Rev. Ecol. Syst. 32: 397-414

Lambers H; Shane MW; Cramer MD; Pearse SJ; Veneklaas EJ. (2006) Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. Annals of Botany. 98: 693-713

Lambert JB; Shawl CE; Poinar GO Jr.; Santiago-Blay JA. (1999) Classification of modern resins by solid state nuclear magnetic resonance spectroscopy. Bioorganic Chemistry. 27: 409-433

Levy Y; Syvertsen JP. (1983) Effect of drought stress and vesicular-arbuscular mycorrhiza on citrus transpiration and hydraulic conductivity of roots. New Phytologist. 93: 61-66

Lewis D; Quirk JP. (1967) Phosphate diffusion in soil and uptake by plants. IV. Computed uptake by model roots as a result of diffusive flow. Plant and Soil. 26: 454-468

Malajczuk N; Cromack K Jr. (1982) Accumulation of calcium oxalate in the mantle of ectomycorrhizal roots of *Pinus radiata* and *Eucalyptus marginata*. New Phytologist. 92: 527-531

Martín MLS; Azcón R; Barea JM; Soriano AP; Goldaracena IM; Piedra AP. (2006) Reduction of the juvenile period of new olive plantations through the early application of mycorrhizal fungi. Soil Science. 171: 52-58

Marx DH. (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology. 59: 153-163

Marx DH; Kenney DS. (1982) Production of ectomycorrhizal fungus inoculum. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 131-146

Massicotte HB; Molina R; Tackaberry LE; Smith JE; Amaranthus MP. (1999) Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. Can. J. Bot. 77: 1053-1076

McGee PA; Bullock S; Summerell BA. (1999) Structure of mycorrhizae of the Wollemi pine (*Wollemia nobilis*) and related Araucariaceae. Aust. J. Bot. 47: 85-95

McGonigle TP; Miller MH; Evans DG; Fairchild GL; Swan JA. (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist. 115: 495-501

McKendrick SL; Leake JR; Read DJ. (2000) Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. New Phytologist. 145: 539-548

Mejstrik VK. (1972) Vesicular-arbuscular mycorrhizas of the species of a *Molinietum coeruleae* L.I. association: the ecology. New Phytologist. 71: 883-890

Menge JA; Timmer LW. (1982) Procedures for inoculation of plants with vesicular-arbuscular mycorrhizae in the laboratory, greenhouse, and field. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 59-68

Mikola P. (1965) Studies on ectendotrophic mycorrhiza of pine. Acta Forestalia Fennica. 79: 1-56

Molina R. (1982) Use of the ectomycorrhizal fungus *Laccaria laccata* in forestry. I. Consistency between isolates in effective colonization of containerized seedlings. Can. J. For. Res. 12: 469-473

Molina R; Palmer JG. (1982) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 115-130

Moreira-Souza M; Trufem SFB; Gomes-da-Costa SM; Cardoso EJBN. (2003) Arbuscular mycorrhizal fungi associated with *Araucaria angustifolia* (Bert.) O. Ktze. Mycorrhiza. 13: 211-215

Morrison TM; English DA. (1967) The significance of mycorrhizal nodules of *Agathis australis*. New Phytologist. 66: 245-250

Newman EI; Reddell P. (1987) The distribution of mycorrhizas among families of vascular plants. New Phytologist. 106: 745-751

Newsham KK; Fitter AH; Watkinson AR. (1995) Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. Journal of Ecology. 83: 991-1000

Ning J; Cumming JR. (2001) Arbuscular mycorrhizal fungi alter phosphorus relations of broomsedge (*Andropogon virginicus* L.) plants. Journal of Experimental Biology. 52: 1883-1891

Offord C; Meagher P. (2006) Wollemi Pine: from the wild to the world. Chronica Horticulturae. 46: 10-13

Ohtomo R; Saito M. (2005) Polyphosphate dynamics in mycorrhizal roots during colonization of an arbuscular mycorrhizal fungus. New Phytologist. 167: 571-578

Orlovich DA; Ashford AE. (1993) Polyphosphate granules are an artefact of specimen preparation in the ectomycorrhizal fungus *Pisolithus tinctorius*. Protoplasma. 173: 91-102

Peakall R; Ebert D; Scott LJ; Meagher PF; Offord CA. (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). Molecular Ecology. 12: 2331-2343

Peat HJ; Fitter AH. (1993) The distribution of arbuscular mycorrhizas in the British flora. New Phytologist. 125: 845-854

Peterson RL; Massicotte HB; Melville LH. (2004) Mycorrhizas: anatomy and cell biology. Ottawa, Ontario: NRC Research Press.

Phillips JM; Hayman DS. (1970) Improved procedure for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society. 55: 158-161

Piché Y; Fortin JA. (1982) Development of mycorrhizae, extraradical mycelium and sclerotia on *Pinus strobus* seedlings. New Phytologist. 92: 211-220

Prabhu V; Biolchini PF; Boyer GL. (1996) Detection and identification of ferricrocin produced by ectendomycorrhizal fungi in the genus *Wilcoxina*. BioMetals. 9: 229-234

Quilambo OA; Weissenhorn I; Doddema H; Kuiper PJC; Stulen I. (2005) Arbuscular mycorrhizal inoculation of peanut in low-fertile tropical soil. I. Host-fungus compatibility. Journal of Plant Nutrition. 28: 1633-1644

Rasmussen N; Lloyd DC; Ratcliffe RG; Hansen PE; Jakobsen I. (2000) ^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. Plant and Soil. 226: 245-253

Redecker D; Kodner R; Graham LE. (2000) Glomalean fungi from the Ordovician. Science. 289: 1920-1921

Redlak K; Dahm H; Ciesielska A; Strzelczyk E. (2001) Enzymatic activity of ectomycorrhizal fungi. Biology and Fertility of Soils. 33: 83-90

Riffle JW; Maronek DM. (1982) Ectomycorrhizal inoculation procedures for greenhouse and nursery studies. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 147-155

Rillig MC; Wright SF; Eviner VT. (2002) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant and Soil. 238: 325-333

Robinson D. (1994) The response of plants to non-uniform supplies of nutrients. New Phytologist. 127: 635-674

Russell AJ; Bidartondo MI; Butterfield BG. (2002) The root nodules of the Podocarpaceae harbour arbuscular mycorrhizal fungi. New Phytologist. 156: 283-295

Sanders FE; Tinker PB. (1973) Phosphate flow into mycorrhizal roots. Pesticide Science. 4: 385-395

Scales PF; Peterson RL. (1991) Structure and development of *Pinus banksiana – Wilcoxina* ectendomycorrhizae. Can. J. Bot. 69: 2135-2148

Schüβler A; Schwarzott D; Walker C. (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycological Research. 105: 1413-1421

Siemens JA; Zwiazek JJ. (2008) Root hydraulic properties and growth of balsam poplar (*Populus balsamifera*) mycorrhizal with *Hebeloma crustuliniforme* and *Wilcoxina mikolae* var. *mikolae*. Mycorrhiza. 18: 393-401

Simard SW; Perry DA; Jones MD; Myrold DD; Durall DM, Molina R. (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. Nature. 388: 579-582

Simon L; Bousquet J; Lévesque RC; Lalonde M. (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature. 363: 67-69

Smith SE; Read DJ. (1997) Mycorrhizal symbiosis. 2nd Ed. San Diego, California: Academic Press, Inc.

Snellgrove RC; Splittstoesser WE; Stribly DP; Tinker PB. (1982) The distribution of carbon and the demand of the fungal symbiont in leek plants with vesicular-arbuscular mycorrhizas. New Phytologist. 92: 75-87

Stribley DP; Tinker PB; Snellgrove RC. (1980) Effects of vesicular-arbuscular mycorrhizal fungi on the relations of plant growth, internal phosphorus concentration and soil phosphate analyses. Journal of Soil Science. 31: 655-672

Sutton JC. (1973) Development of vesicular-arbuscular mycorrhizae in crop plants. Can. J. Bot. 51: 2487-2493

Takanishi I; Ohtomo R; Hayatsu M; Saito M. (2009) Short-chain polyphosphate in arbuscular mycorrhizal roots colonized by *Glomus* spp.: a possible phosphate pool for host plants. Soil Biology & Biochemistry. 41: 1571-1573

Tawaraya K; Watanabe S; Vierheilig H; Wagatsuma T. (2007) Formation of appressoria by the arbuscular mycorrhizal fungus *Gigaspora margarita* on roots of *Allium cepa* is linked with root age. Mycoscience. 48: 305-308

Tedersoo L; Hansen K; Perry BA; Kjøller R. (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytologist. 170: 581-596

Thomson VP; Leishman MR. (2004) Survival of native plants of Hawkesbury Sandstone communities with additional nutrients: effect of plant age and habitat. Aust. J. Bot. 52: 141-147

Ursic M; Peterson RL; Husband B. (1997) Relative abundance of mycorrhizal fungi and frequency of root rot on *Pinus strobus* seedlings in a southern Ontario nursery. Can. J. For. Res. 27: 54-62

Williams PG. (1992) Axenic culture of arbuscular mycorrhizal fungi. Methods in Microbiology. 24: 203-220

Wright SF; Upadhyaya A. (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Science. 161: 575-586

Yang CS; Korf RP. (1985) A monograph of the genus *Tricharina* and of a new, segregate genus, *Wilcoxina* (Pezizales). Mycotaxon. 24: 467-531

Yang CS; Wilcox HE. (1984) An E-strain ectendomycorrhiza formed by a new species, *Tricharina mikolae*. Mycologia. 76: 675-684

Yu TEJ-C; Egger KN; Peterson RL. (2001) Ectendomycorrhizal associations – characteristics and functions. Mycorrhiza. 11: 167-177

Zandavalli RB; Dillenburg LR; de Souza PVD. (2004) Growth response of *Araucaria angustifolia* (Araucariaceae) to inoculation with the mycorrhizal fungus *Glomus clarum*. Applied Soil Ecology. 25: 245-255

Chapter 2: Inoculation of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) with Mycorrhizal Fungi Under Controlled Conditions[1]

2.1 Introduction

Mycorrhizal fungi are ubiquitous organisms that form mutualistic symbiotic associations with the roots of over 90 % of terrestrial plants. In a mycorrhizal association, the fungus provides the plant with nutrients and other benefits in exchange for a portion of the plant's photosynthate (Smith and Read, 1997). The most widely studied role of mycorrhizal fungi is scavenging mineral nutrients such as nitrogen and phosphorus for the host plant (Newsham et al., 1995). For this reason, colonization by mycorrhizal fungi may benefit plant health by improving nutrient status, especially for plants inhabiting soils with low nutrient availability (Hardie and Leyton, 1981). Improved nutrition is of particular importance in the establishment and long-term survival of endangered or threatened plant species, and mycorrhizal fungi may be crucial in enhancing the survival capacity of plants in danger of extinction.

The Wollemi Pine (Araucariaceae) is an endangered tree that was once thought to be extinct but was discovered in 1994 in a secluded area of Wollemi National Park in southeastern Australia (Jones et al., 1995). Since then, research on Wollemi Pine has been driven by conservation and propagation efforts. An important research area that has received little attention is the mycorrhizal colonization of Wollemi Pine roots. McGee et al. (1999)

[1] A version of this chapter will be submitted for publication. Biggs LE; Murch SJ; Durall DM. Inoculation of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) with Mycorrhizal Fungi Under Controlled Conditions.

found evidence of both arbuscular mycorrhizas and ectendomycorrhizas in Wollemi Pine roots in the field. In general, arbuscular mycorrhizal associations are the most widespread type (Peat and Fitter, 1993), and have previously been detected in plants belonging to the Araucariaceae (e.g. Morrison and English, 1967). Ectendomycorrhizas, which are relatively uncommon associations, typically form between the fungal genus *Wilcoxina* and the plant genera *Pinus* and *Larix* (Yang and Korf, 1985; Yu et al., 2001). The formation of two distinct types of mycorrhizal associations found in Wollemi Pine roots is a unique occurrence for the family Araucariaceae. The next step in this research area is to quantify any benefits attributed to the Wollemi Pine from the different types of associations, and then apply that information to conservation strategies.

The overall objective of the study was to determine, in two inoculation experiments, whether arbuscular mycorrhizal fungi or *Wilcoxina mikolae* will colonize Wollemi Pine roots in a controlled setting. The first inoculation experiment incorporated three arbuscular mycorrhizal fungi and *Wilcoxina mikolae* obtained from culture collections, while the second experiment involved inoculating Wollemi Pines with leek pot culture roots colonized by one of two arbuscular mycorrhizal fungi. The specific objectives of the research were (a) to compare and quantify mycorrhizal fungal colonization in roots of Wollemi Pine plants inoculated with *Glomus intraradices*, *G. clarum*, *Scutellospora heterogama*, or *W. mikolae*, (b) to measure the effects of the mycorrhizal inoculation on plant growth, and (c) to measure the effects of the mycorrhizal inoculation on plant tissue nutrient composition.

2.2 Materials and Methods

2.2.1 Inoculation of Wollemi Pines with Mycorrhizal Fungi

Wollemi Pines were inoculated with mycorrhizal fungi in two separate experiments. In the first experiment, the plants were inoculated with *Wilcoxina mikolae* or one of three species of arbuscular mycorrhizal fungi. All fungal material used in the first set of inoculations was obtained from culture collections (section 2.2.2.1). The second experiment incorporated pot cultures of leek (*Allium porrum* L.) colonized with one of two arbuscular mycorrhizal fungi as an inoculum source for the Wollemi Pines (section 2.2.3.1).

2.2.1.1 Plant Material

The plants used in both inoculation experiments were propagated from Wollemi Pine cuttings in Australia, which entered Canada in 2005 in accordance with CFIA inspections, permits, and regulations. The two year-old plants were transferred from Nordic Nurseries in Abbotsford, British Columbia to UBC Okanagan in Kelowna, British Columbia in July 2007. Upon arrival at UBC Okanagan, the plants were growing in peat-based substrate in 15 cm diameter pots and the shoot heights ranged from 30 to 50 cm. The plants were acclimated in a Conviron E15 growth chamber at 27 ^{o}C/20 ^{o}C day/night with a 16 hour photoperiod of 200 μmol $m^{-2}s^{-1}$ at pot height.

Prior to starting the inoculation experiments, the root systems of six randomly selected experimental plants were sampled and inspected for mycorrhizal colonization. A 4 mm cork borer was used to obtain root samples, which were rinsed to remove adhering soil particles. The washed roots were viewed under a Nikon SMZ1000 dissecting microscope

and photographed using a Nikon DXM1200 digital camera and Nikon Act-1 software (Version 2.20, LEAD Technologies, Inc). Using fine forceps, the outer layers of a subset of the roots were removed and mounted on glass slides in 1:1 (v/v) lactoglycerol (Newsham et al., 1994). The slides were viewed using a Nikon Eclipse E800 light microscope to look for fungal structures on the root surface.

The remaining root samples were put in staining baskets lined with nylon mesh and stained with chlorazol black E stain according to Brundrett et al. (1984). In brief, the staining baskets were immersed in 10 % potassium hydroxide and autoclaved on the slow exhaust cycle for 15 minutes. Once the baskets had cooled slightly, the roots were bleached with 30 % hydrogen peroxide for 20 minutes. The baskets were then rinsed with several changes of tap water and deionized water, and treated with 1 M hydrochloric acid for 15 minutes. The roots were heated in chlorazol black E stain (consisting of equal parts of glycerol, 85 % lactic acid, and 0.1% (w/v) chlorazol black E solution) at 90 ^{o}C for one hour. Following the staining, the roots were destained overnight in glycerol. To look for internal fungal structures, the stained roots were viewed using a light microscope and photographed as described above.

2.2.2 Inoculation of *W. nobilis* with Arbuscular Mycorrhizal and *Wilcoxina mikolae* Inoculum Obtained from Culture Collections

2.2.2.1 Fungal Material

Three isolates of arbuscular mycorrhizal fungi were selected for use in the inoculation experiments, and were obtained from the International Culture Collection of Arbuscular and

Vesicular-Arbuscular Mycorrhizal Fungi (INVAM; Morgantown, West Virginia, U.S.A.). The species obtained from this source were *Glomus clarum* Nicol. & Schenck INVAM BR143A, *Glomus intraradices* Schenck & Smith INVAM UT126, and *Scutellospora heterogama* (Nicol. & Gerd.) Walker & Sanders INVAM NY320. These fungi were received as whole inoculum composed of spores, hyphae, and fragments of colonized *Sorghum sudanense* (Piper) Stapf. roots grown in Terra-green substrate (Oil Dri Corporation of America, Chicago, Illinois, U.S.A.). The inoculum remained sealed and was stored at 4 ^{o}C for one week prior to use, as recommended by INVAM.

The fourth species used as inoculum, *Wilcoxina mikolae* var. *mikolae* Yang & Korf UAMH 6844 was obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH; Edmonton, Alberta, Canada). The fungus was sent as a slant culture growing on potato dextrose agar (PDA). The culture was left in the slant tube at room temperature for two weeks and was then sub-cultured into liquid modified Melin-Norkans (MMN) medium at pH 5.5, as per Marx (1969). Two hundred and fifty mL quantities of the medium were transferred into Erlenmeyer flasks each containing 10 to 15 pieces of broken glass measuring approximately 2 by 2 cm, and the flasks were autoclaved for 40 minutes. In a laminar flow hood, one 0.5 by 0.5 cm square from the PDA slant was aseptically transferred into each flask. The flasks were covered with foam plugs, foil, and parafilm and removed from the hood. The fungi were grown in static culture at room temperature under regular fluorescent light and indirect sunlight and were transferred into fresh media every eight weeks. The flasks were shaken on a weekly basis to fragment the actively growing hyphae for more rapid fungal growth (Molina and Palmer, 1982).

2.2.2.2 Experimental Design, Planting, and Growing of Plants

The first inoculation experiment was performed in October 2007. Using a completely randomized design, four fungal treatments and one control treatment were each replicated four times for a total of 20 plants. The uninoculated control plants received equal volumes of sterile sand in place of fungal inoculum (Lesica and Antibus, 1990). The arbuscular mycorrhizal inoculum was used as supplied. The *W. mikolae* var. *mikolae* inoculum was prepared by vacuum filtering the contents of four week-old culture flasks and rinsing the hyphae with sterile deionized water. The rinsed hyphae from six culture flasks were combined and homogenized in a Waring blender with a small amount of sterile deionized water, using four one-second high-speed pulses (Marx and Kenney, 1982). The contents of the blender were diluted to 325 mL using sterile deionized water to produce a solution of hyphal inoculum.

The Wollemi Pines were removed from their original pots and replanted in sterile 15 cm diameter pots using 1 L of coconut fiber substrate (CocoTek® Natural Coconut Coir™ block; General Hydroponics Inc, Sebastopol, California, U.S.A.) that had been autoclaved twice for 40 minutes with 24 hours in between. The previous substrate external to the root ball was removed but the root systems were not disturbed in the planting. Seventy-seven mL of the appropriate inoculum or sterile sand was uniformly mixed with the substrate prior to planting, providing an inoculum to substrate ratio of 1:13 (v/v) (Daniels and Skipper, 1982). For the duration of the experiment, the trees were randomized in one Conviron E15 growth chamber maintained at 27 ^{o}C/20 ^{o}C day/night with a 16 hour photoperiod of 250 μmol $m^{-2}s^{-1}$ at pot height. The plants were watered to saturation every five days using lukewarm tap

water. The plant heights from the soil surface to the top of the main growth stem were measured weekly.

2.2.2.3 Root Sampling, Harvesting of Plants, and Observation of Colonization by Mycorrhizal Fungi

Eight weeks following inoculation, root samples were taken from one plant of each of the five treatments. The plants were removed from their pots and fine forceps were used to randomly extract 15 roots 2 cm to 3 cm in length from the perimeter of the root ball. The five plants were immediately repotted and provided with water. The sampled roots were stained using the chlorazol black E staining method (Brundrett et al., 1984), as previously described in section 2.2.1.1. The stained roots were viewed and photographed as before. The percent colonization of each root sample was determined using the magnified intersections method (McGonigle et al., 1990). This method involved using a light microscope with an ocular lens containing vertical and horizontal crosshairs to make six vertical passes of the slide containing all the stained roots from one plant. The presence or absence of fungal structures was noted each time the crosshairs intersected with a root. The percent colonization of the roots was calculated by dividing the number of intersection points containing fungal structures by the total number of intersection points.

All 20 plants were harvested in January 2008, fourteen weeks after inoculation. The roots and shoots were separated at the root crown and the root systems were gently washed in warm water to remove substrate. Fine forceps were used to remove 15 randomly selected roots from each root system. As described above, the roots were stained and viewed, and percent colonization was assessed. The remaining roots and shoots of each plant were put in

separate pre-weighed paper bags and weighed to determine the tissue wet weights. The tissue was oven-dried at 45 ^{o}C to a constant weight. The dry tissue was then ground to pass a 1 mm sieve using a Retsch® SM2000 mill and stored at -20 ^{o}C. A portion of each ground root and shoot tissue sample was sent to CANTEST Ltd in Burnaby, British Columbia for analysis of the following components: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, protein, nitrogen, fat, moisture, ash, carbohydrates, and energy in units of calories and Joules. To measure mineral content in the tissue, the samples were digested using a nitric acid-hydrogen peroxide digestion procedure and analyses were performed using Inductively Coupled Argon Plasma Spectroscopy (ICP). Proximate analyses were performed as described by Horwitz (2000).

2.2.3 Inoculation of *Wollemia nobilis* with Leek Pot Culture Roots Colonized by Arbuscular Mycorrhizal Fungi

2.2.3.1 Pot Culture Inoculation of Leek

To incorporate an inoculum source of known viability and age, mycorrhizal fungal inoculum for the second set of inoculations was derived from pot cultures of leek (*Allium porrum* L. cv. Giant Musselburgh). The leek pot cultures were established in 15 pots containing five fungal treatments and three replicates of each. Three of the fungal treatments used whole inoculum of the same three arbuscular mycorrhizal fungal isolates from INVAM (see section 2.2.2.1), the fourth fungal treatment was *Glomus intraradices* MYCORISE® spore inoculum (Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada), and the

final treatment was an uninoculated control. Each replicate was planted in a sterile 25 cm diameter pot using 2:1 peat/sand mixture (Sun Gro Horticulture Canada Ltd, Seba Beach, Alberta, Canada), which is a commonly used leek substrate (e.g. Amijee et al., 1990). Prior to the plantings, the substrate was autoclaved twice for 40 minutes with 24 hours in between. For the three whole inoculum treatments, the ratio of inoculum to substrate in each pot was 308 mL to 4 L or 1:13 (v/v) (Daniels and Skipper, 1982). For these treatments, the inoculum was placed in a layer about 2 cm below the seeds (Menge and Timmer, 1982) to increase the likelihood of contact with developing roots. For pots receiving spore inoculum, 20 mL of spore solution was added to each pot to yield approximately 8000 spores in 4 L of substrate (Ferguson and Woodhead, 1982), applied in the same layering format as the whole inoculum. The uninoculated control treatments received only 4 L of peat/sand substrate. Prior to planting, the leek seeds were surface-sterilized in 8 % household bleach (5.25 % sodium hypochlorite) for 10 minutes and rinsed thoroughly with sterile distilled water (Geil and Guinel, 2002). Approximately 150 seeds were planted per pot and covered with a thin layer of substrate. The pots were kept on a bench in the UBC Okanagan Arts Greenhouse (Figure 2.1). The leeks were grown at a temperature range of 19 ^{o}C to 26 ^{o}C with a 16 hour photoperiod of 450 μmol $m^{-2}s^{-1}$ at pot height (Hamel et al., 1997), measured with a Li-Cor LI-185A Photometer (LI-COR Biotechnology, Lincoln, Nebraska, U.S.A.). The pots were watered to saturation with deionized water every second day (Hamel et al., 1997).

Figure 2.1 Leek (*Allium porrum*) pot cultures established to multiply propagules of arbuscular mycorrhizal fungi and provide a local source of inoculum for Wollemi Pines.

Four weeks after planting the leek pot cultures, root samples were taken to confirm colonization. Fifteen leeks were uprooted from each pot using fine forceps, and the root portions were separated into fragments 2 to 3 cm in length. The root samples were put in staining baskets lined with nylon mesh and stained with chlorazol black E, as described by Brundrett et al. (1984) but with some slight modifications due to the delicacy of the roots. Briefly, the staining baskets were immersed in 10 % potassium hydroxide and autoclaved on the slow exhaust cycle for five minutes. The baskets were cooled and rinsed with several changes of tap water and deionized water, and then heated in chlorozal black E stain (consisting of equal parts of glycerol, 85 % lactic acid, and 0.1 % (w/v) chlorazol black E

solution) at 90 ^{o}C for one hour. The baskets were submerged in glycerol overnight to destain the roots. The roots were viewed and photographed as previously described (Figure 2.2). The magnified intersections method was used to determine the percent colonization of the root samples (McGonigle et al., 1990).

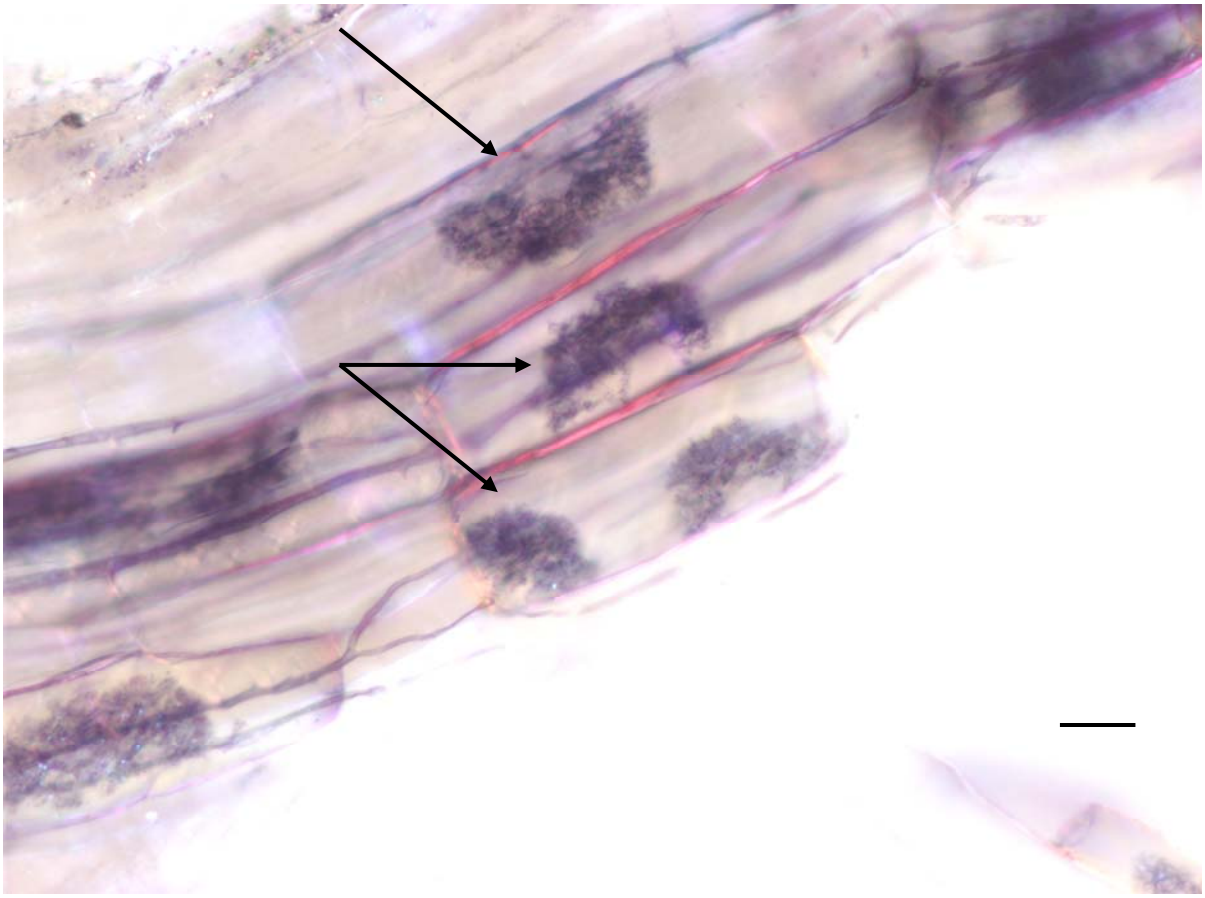

Figure 2.2 Leek root from pot culture stained with chlorazol black E and viewed under the 20X objective of a light microscope (Bar = 20 μm). Note the intracellular arbuscules formed by the fungus *Glomus intraradices* (indicated by the arrows).

2.2.3.2 Experimental Design, Planting, and Growing of Plants

In May 2008, Wollemi Pines were inoculated with inoculum obtained from the leek pot cultures. The plant material was obtained from the same source and stored in the same

manner as described in the first inoculation study (section 2.2.1.1). The experiment was set up as a completely randomized design consisting of three treatments and four replicates of each, for a total of 12 plants. Two of the treatments were inoculated with leek pot culture roots colonized by *Glomus intraradices* or *G. clarum* fungi from INVAM. Only pot cultures exhibiting root colonization greater than 80 % were used as inoculum for the fungal treatments. Because none of the *Scutellospora heterogama* pots displayed high enough colonization, this fungal treatment was omitted from the experiment. To prepare the inoculum, the colonized leek roots were chopped into 1 cm fragments. The third treatment, the control, received chopped leek roots that had not been inoculated and, therefore, were confirmed to be not colonized.

Each Wollemi Pine was planted in a sterile 20 cm diameter pot using 2 L of autoclaved 2:1 peat/sand substrate. Approximately 10 g fresh weight, or 200 mL, of chopped colonized leek roots combined from all suitable leek pots were uniformly mixed with the substrate immediately before planting, providing an inoculum to substrate ratio of 1:10 (v/v). This ratio is greater than typically recommended (Daniels and Skipper, 1982), but has been used in some inoculation studies involving leek roots (e.g. Vimard et al., 1999). In addition to the leek root pieces, colonized leek plants from the pot cultures were planted in each experimental pot as an additional inoculum source (Figure 2.3). Each pot received a total of 25 leek plants colonized by the appropriate fungus, or not colonized for the control treatment, which were planted in clusters of five as close as possible to the Wollemi Pine root system. As a positive control, 20 surface-sterilized leek seeds were also planted in each experimental pot. Therefore, each of the twelve pots contained one Wollemi Pine, chopped leek roots, 25 leek plants, and 20 leek seeds. For the duration of the experiment the pots were randomized

in one Conviron CMP3244 growth chamber maintained at 26 ^{o}C/20 ^{o}C day/night with a 16 hour photoperiod of 250 µmol $m^{-2}s^{-1}$ at pot height. The plants were watered to saturation once per week using lukewarm tap water. Plant heights from the soil surface to the top of the main shoot were measured on a weekly basis.

Figure 2.3 Wollemi Pines inoculated with chopped leek roots and planted with twenty-five germinated leeks as an additional inoculum source. Twenty leek seeds were also planted in each experimental pot.

2.2.3.3 Root Sampling, Harvesting of Plants, and Observation of Colonization by Mycorrhizal Fungi

Ten weeks after planting, one plant was randomly selected from each of the three treatments for harvest and root analysis. To prevent overlooking colonization, all of the roots from each plant were examined. Roots were subsampled, stained, and examined as described in section 2.2.1.1.

The remaining plants from the second experimental set were harvested 18 weeks after inoculation. In addition to examining the roots as described above, the shoots were put in pre-weighed paper bags and weighed to determine wet weight. The shoot tissue was dried, ground, and analyzed as described in section 2.2.2.3.

2.2.4 Data Analysis

For both experiments, total growth was calculated for each plant as the difference between the plant height measured just prior to harvesting and the initial plant height. Averaging the weekly increases in plant height produced growth rates for each plant. Treatment means of total growth and growth rate were calculated.

To test for differences between control treatments and mycorrhizal treatments in total growth, growth rates, and tissue nutrient composition, the Generalized Linear Model (GLM) Procedure was used to perform a one-way analysis of variance (ANOVA) on treatment means with a posteriori means separation using Duncan's Multiple Range test (DMRT) (SAS software; SAS Institute Inc, Cary, North Carolina, U.S.A.). This analysis was performed separately for the two inoculation experiments, using nutrient analysis data for both the shoot

and root tissue data from the first experiment (each n = 20) and the shoot tissue data from the second experiment (n = 9).

2.3 Results

2.3.1 Colonization

In the pot cultures, leek seeds began germinating within three days and the roots were colonized within four weeks (Figure 2.2). Pots inoculated with *G. intraradices* inoculum from INVAM exhibited 80 to 95% root colonization, while the *G. intraradices* spore inoculum resulted in 7 to 44 % colonization in roots that were sampled. Roots sampled from pots inoculated with *G. clarum* inoculum from INVAM were between 40 and 93 % colonized by the fungus. Pots inoculated with *S. heterogama* inoculum from INVAM showed 0 to 33 % root colonization and were therefore not included as an inoculum treatment for Wollemi Pines. Roots were also examined from the pots that did not receive inoculum and were found to be devoid of colonization. An important observation from this study was that all of the leek plants transplanted into the Wollemi Pine pots became brown and died within the first few weeks of the experiment (Figure 2.4). Also, germination of leek seeds was not evident in any of the experimental pots.

Figure 2.4 A Wollemi Pine from the second inoculation experiment, six weeks after being inoculated. Note that all of the leek plants died (indicated by the arrows), and leek seed germination was not evident.

In both inoculation experiments, no mycorrhizal fungal structures were observed in the stained roots that were examined by light microscopy. Therefore, for both experiments, of the roots examined, the mycorrhizal colonization was 0 % for each plant of each treatment. However, all of the inspected roots were colonized to varying extents by endophytic fungal structures, including septate hyphae and intracellular sclerotia, as shown in Figure 2.5.

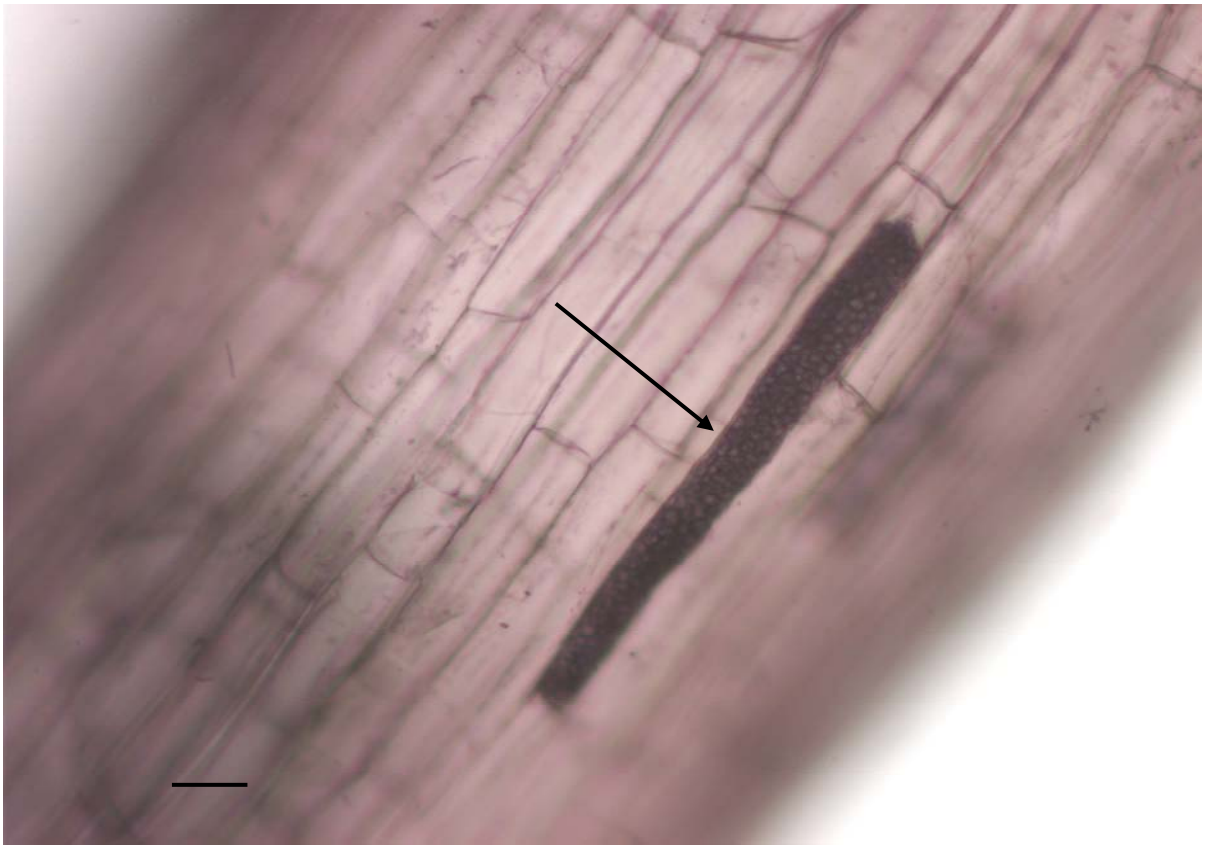

Figure 2.5 A Wollemi Pine (*Wollemia nobilis*) root from the second inoculation experiment stained with chlorazol black E and viewed under the 20X objective of a light microscope (Bar = 20 μm). Note the intracellular fungal sclerotium (indicated by the arrow).

2.3.2 Growth and Nutrient Composition of Experimental Plants

2.3.2.1 Response of *Wollemia nobilis* Inoculated with Arbuscular Mycorrhizal Fungi and *Wilcoxina mikolae* Obtained from Culture Collections

All of the Wollemi Pines survived to the end of the experiment. There were no differences in total growth increment ($P = 0.58$, $F = 0.73$, $df = 19$) and growth rate ($P = 0.59$, $F = 0.72$, $df = 19$) between the control treatment and the inoculated plants (Table 2.1). The

analysis of *W. nobilis* tissue exposed to different fungal inoculants revealed several interesting responses, especially given that the seedlings were not colonized. Significantly more iron ($P = 0.013$, F = 6.51, df = 19) and calcium ($P = 0.042$, F = 3.49, df = 19) accumulated in shoot tissues of plants exposed to *S. heterogama*, *G. clarum*, and the uninoculated controls than was observed in seedlings exposed to *W. mikolae* or *G. intraradices* (Figures 2.6 and 2.7). Inoculation with *G. intraradices* also significantly reduced the total mineral accumulation measured as ash as compared to the *S. heterogama* treatment and the uninoculated control plants ($P = 0.039$, F = 4.52, df = 19) (Figure 2.8). Moisture content in roots also significantly differed between treatments. Inoculation with *G. clarum* increased moisture content in roots, while root moisture content was less for plants exposed to *S. heterogama* as compared to the other treatments ($P = 0.019$, F = 6.25, df = 19) (Figure 2.9). Root nitrogen ($P = 0.089$, F = 2.44, df = 19) and protein ($P = 0.0075$, F = 8.61, df = 19) accumulation was significantly reduced in plants exposed to *W. mikolae* var. *mikolae* compared to plants exposed to *G. clarum* or *S. heterogama* (Figures 2.10 and 2.11). None of the other parameters that were measured in the root and shoot tissue differed significantly amongst the inoculation treatments.

Table 2.1 Total shoot growth increment and growth rates by treatment in Experiment 1 over the 14 week period. Note that all mean treatment growth increments and growth rates did not significantly differ by one-way ANOVA at $\alpha = 0.05$. *P* values for the one-way ANOVAs are given.

Treatment	Total shoot growth increment (cm) (mean +/- std error)	Growth rate (cm/week) (mean +/- std error)
Wilcoxina mikolae var. *mikolae*	2.40 +/- 0.31	0.13 +/- 0.02
Glomus intraradices	4.48 +/- 2.10	0.35 +/- 0.19
Scutellospora heterogama	2.58 +/- 1.13	0.19 +/- 0.10
G. clarum	4.85 +/- 1.25	0.32 +/- 0.12
Uninoculated control	3.48 +/- 0.93	0.20 +/- 0.04
	P = 0.58	*P* = 0.59

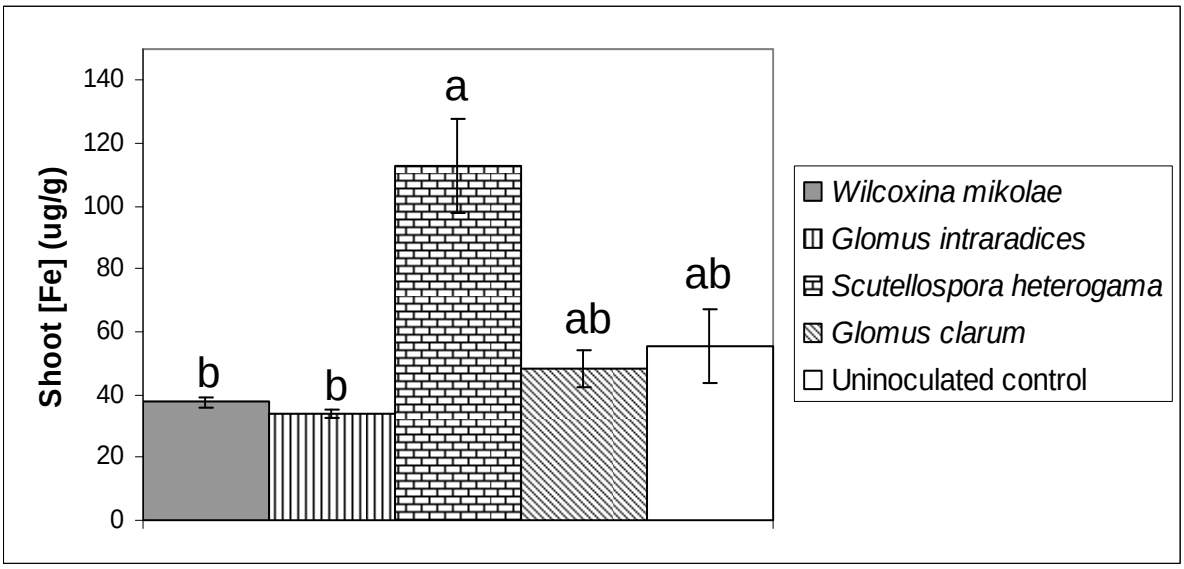

Figure 2.6 Shoot iron concentration in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

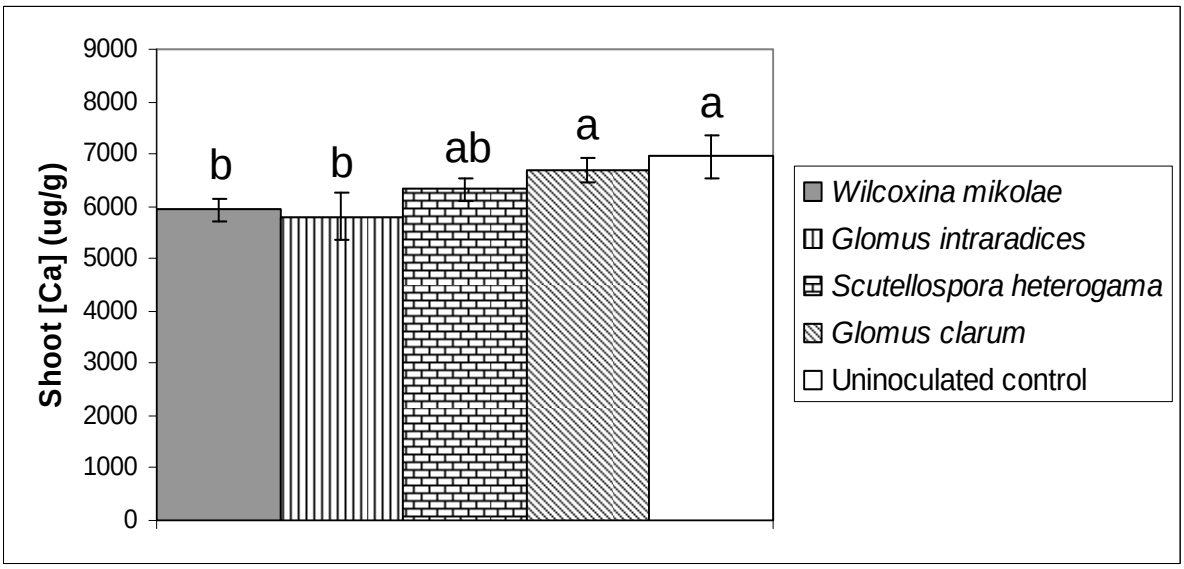

Figure 2.7 Shoot calcium concentration in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

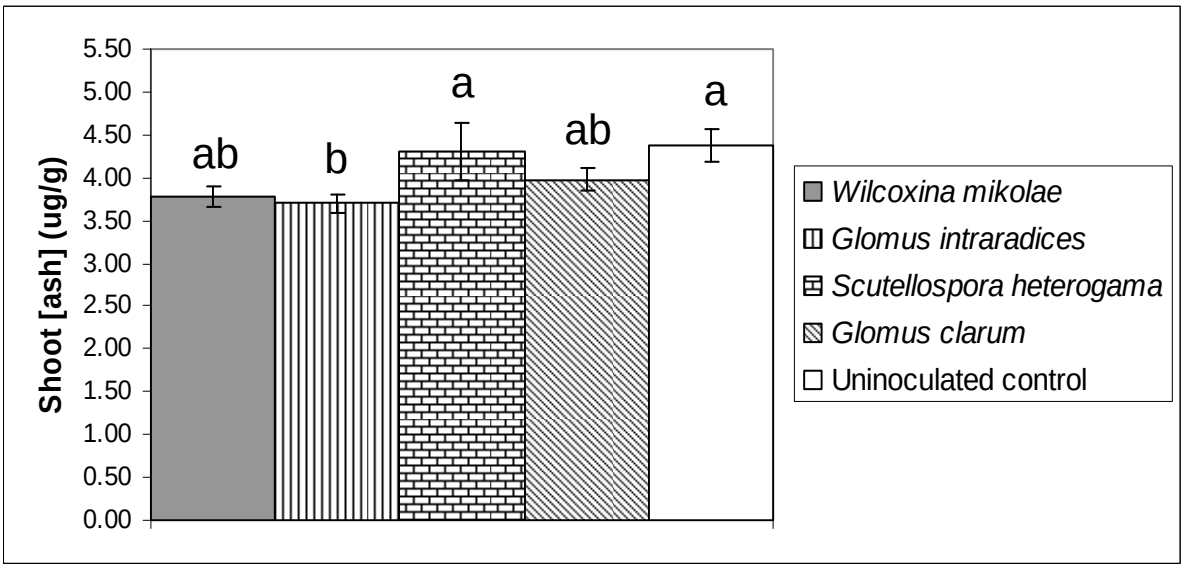

Figure 2.8 Shoot ash concentration in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

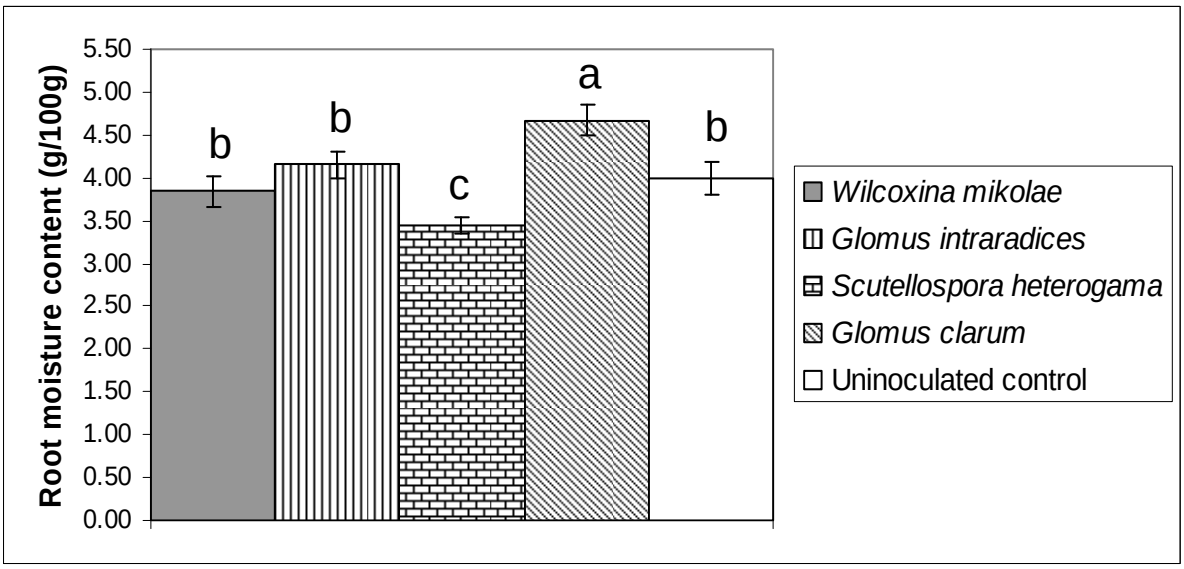

Figure 2.9 Root moisture content in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

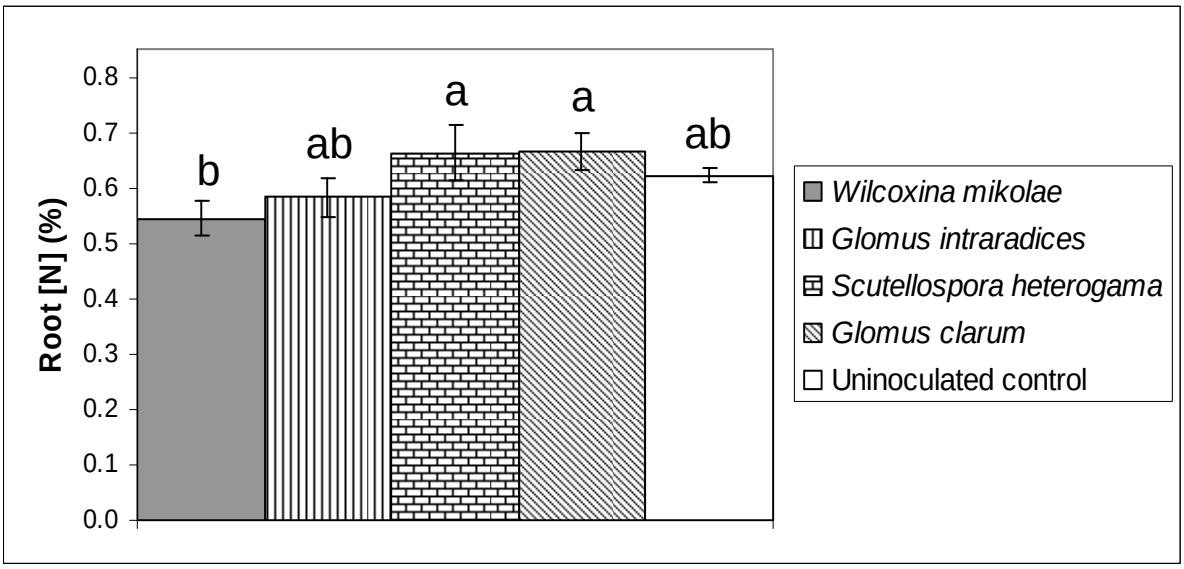

Figure 2.10 Root nitrogen concentration in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

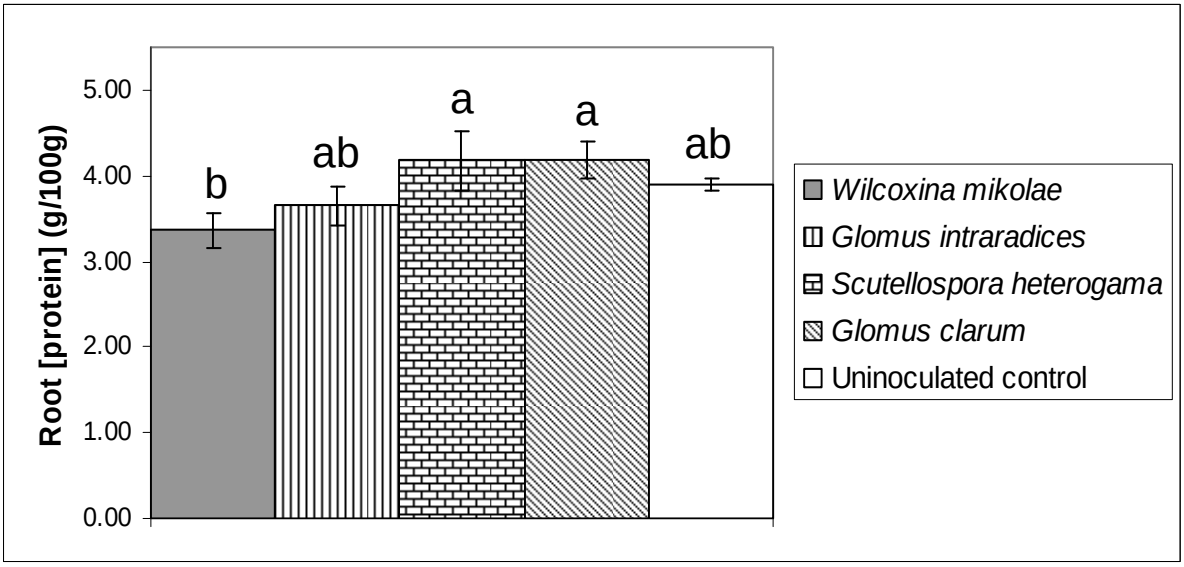

Figure 2.11 Root protein content in *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

Expressing the data as total ion or nutrient content per plant also revealed important trends. Inoculation with *S. heterogama* significantly increased the iron content of shoot tissues as compared to the controls, while plants exposed to *W. mikolae* var. *mikolae* and *G. clarum* had reduced shoot iron content ($P = 0.012$, $F = 6.70$, $df = 19$) (Figure 2.12). The *G. intraradices* treatment significantly reduced the total shoot sodium content as compared to the controls ($P = 0.036$, $F = 4.57$, $df = 19$) (Figure 2.13).

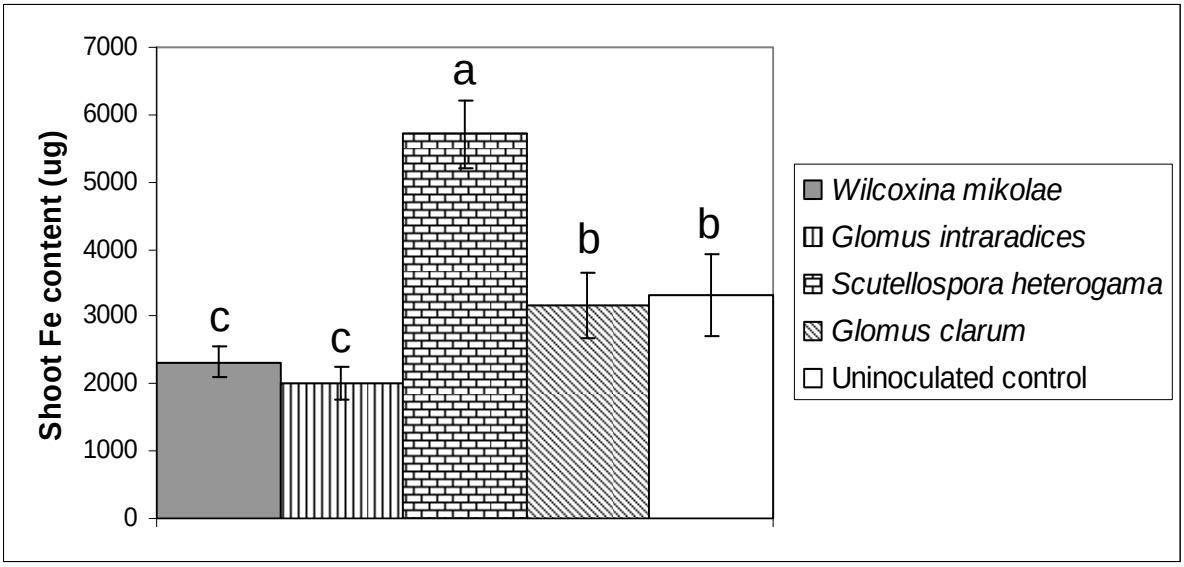

Figure 2.12 Total iron content in shoot tissue of *Wollemia nobilis* plants inoculated with mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

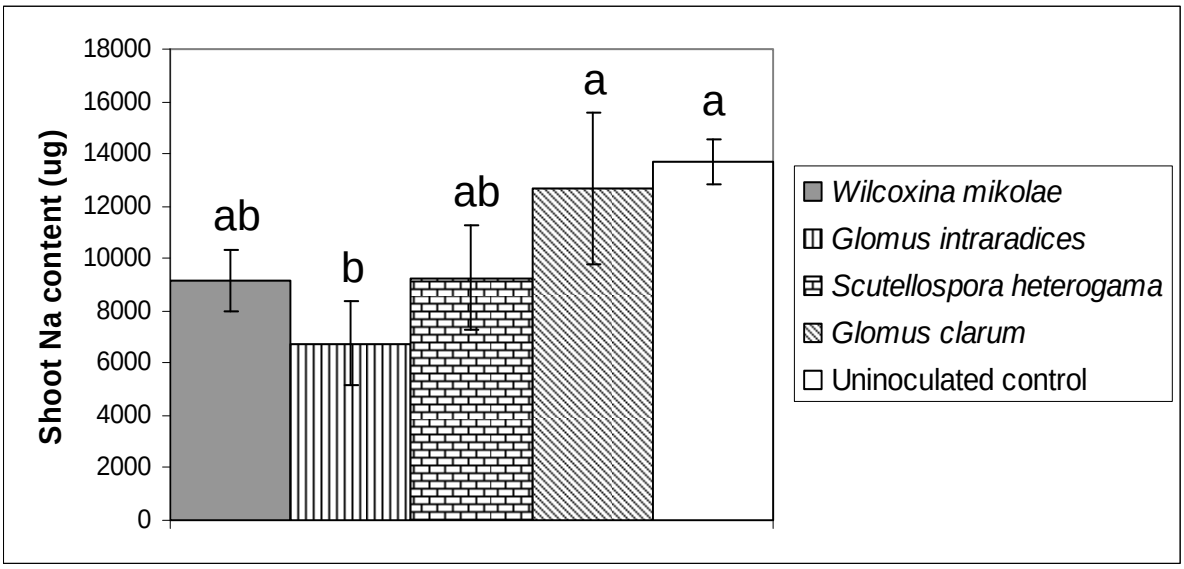

Figure 2.13 Total sodium content in shoot tissue of *Wollemia nobilis* plants inoculated with mycorrhizal fungi Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

2.3.2.2 Response of *Wollemia nobilis* Inoculated with Leek Pot Culture Roots Colonized with Arbuscular Mycorrhizal Fungi

As in the first set of inoculations, all of the Wollemi Pines survived for the duration of the experiment. Significant differences were observed in the total shoot growth increments ($P = 0.0022$, F = 6.76, df = 8) and growth rates ($P = 0.0024$, F = 6.81, df = 8) amongst the inoculation treatments (Table 2.2). Inoculation of the Wollemi Pines with *G. clarum* significantly reduced both the growth increment and the growth rate as compared to the controls and to seedlings inoculated with *G. intraradices*. There were no significant differences between the fungal treatments and the control treatment for any of the nutrient or mineral parameters that were measured in the second study of inoculated *W. nobilis* plants (all $P > 0.05$; data not shown).

Table 2.2 Total growth and growth rates by treatment in Experiment 2 over the 18 week period. Growth increment and growth rate means with different letters are significantly different by Duncan's Multiple Range test at $\alpha = 0.05$. *P* values for the one-way ANOVAs are given.

Treatment	Total shoot growth increment (cm) (mean +/- std error)	Growth rate (cm/week) (mean +/- std error)
Glomus intraradices	3.63 +/- 1.22[a]	0.22 +/- 0.09[a]
G. clarum	2.00 +/- 0.25[b]	0.11 +/- 0.02[b]
Uninoculated control	4.30 +/- 1.18[a]	0.24 +/- 0.07[a]
	P = 0.0022	*P* = 0.0022

2.4 Discussion

Previous studies on the mycorrhizal colonization of *W. nobilis* under laboratory conditions have not been conducted. McGee et al. (1999) examined Wollemi Pine roots from both the natural population and botanic gardens in Australia and observed arbuscular mycorrhizal colonization and one instance of ectendomycorrhizal colonization on the roots of mature trees (McGee et al., 1999). In the current study, mycorrhizal colonization was not detected in Wollemi Pine roots in either of the two approaches. There are several possible explanations for the results. First, while adult Wollemi Pines from the wild were colonized with mycorrhizal fungi, earlier studies with botanic garden specimens of a similar age to those used in the current studies did not detect colonization (McGee et al., 1999). In

addition, the greenhouse and growth chamber environments used to grow Wollemi Pines cannot replicate the environmental parameters of wild plants in their native habitat, particularly with respect to the nutrient dynamics of the growth substrate. It has been demonstrated that plants growing in soils with adequate or higher than adequate nutrient levels, particularly of phosphorus and nitrogen, are less likely to form associations with mycorrhizal fungi than plants growing in nutrient-deficient conditions (Johansen et al., 1994; White and Charvat, 1999; Blanke et al., 2005). Therefore, although not quantified in the current study, the nutrient content of the growth substrate may have affected the experimental outcome.

Another consideration is the suitability and viability of the mycorrhizal fungal inoculum used in the experiments. In the first inoculation experiment, no positive control was included to ensure that the inoculum was capable of root colonization. In the second inoculation experiment, the disappearance of leeks and lack of leek seed germination also eliminated the positive control component of the experiment. In addition, mycorrhizal fungal symbiont specificity has not been determined for Wollemi Pines. A relatively small group of fungi form arbuscular mycorrhizas with a relatively large group of plants, making the association non-specific (Peterson et al., 2004). However, it has been shown that combinations of local plants and fungi produce the most prominent symbiotic responses (Klironomos, 2003), and there is evidence of molecular interactions between symbionts that may affect specificity (Sanders, 2003). *Glomus clarum* was chosen for this experiment because it has been used to successfully inoculate other plants belonging to the Araucariaceae (Zandavalli et al., 2004), while *G. intraradices* has been shown to colonize plants in disturbed and nutrient-deficient Australian soils (Duponnois and Plenchette, 2003).

Also, *Glomus* species tend to be the dominant mycorrhizal fungi on Australian tropical plant roots in both natural and disturbed sites (Brundrett et al., 1999). The third arbuscular mycorrhizal fungal species used, *S. heterogama*, sporulates readily in acidic and tropical soil types (Brundrett et al., 1999; Wu et al., 2002) and flourishes at low to moderate soil phosphorus concentrations (de Miranda and Harris, 1994). *Wilcoxina mikolae* was used because it is known to form ectendomycorrhizas with receptive plant species in disturbed and nutrient-deficient soils (Yang and Korf, 1985). Selection of fungal isolates was limited by both availability and transferability into Canada. All of the isolates were collected in North America; the *W. mikolae* var. *mikolae* culture was originally collected from North Carolina and the three arbuscular mycorrhizal fungal cultures were submitted to INVAM by a laboratory in Utah. Since the fungi used in the current study were not Australian isolates, they may have been incompatible with Wollemi Pines.

The death of all the transplanted and seeded leeks was an important result. It is possible that the leeks were unable to grow in the substrate or could not compete with the Wollemi Pines for nutrients, or it is possible that the Wollemi Pines produced allelopathic chemicals that appear to have been toxic to either the leek plants or the fungi themselves. Allelopathy is described as the suppression of growth of a plant due to phytotoxins released by another, and has been well-documented in gymnosperms, including some examples in the Araucariaceae (Singh et al., 1999). Therefore, there may be a chemical mechanism employed by Wollemi Pines to suppress seed germination and restrain growth for some plants. There is no evidence of allelopathic potential in Wollemi Pines to date, but this will be investigated in future studies.

There were some significant differences in nutrient composition between the fungal treatments and the control treatments in the first inoculation experiment, despite a lack of mycorrhizal colonization observed in the roots. The most likely explanation is that the inoculum affected the plant roots without colonization taking place. Several studies using both arbuscular mycorrhizal and ectomycorrhizal inoculum have shown that inoculation can induce measurable changes in plants in the absence of colonization. For example, Scagel (2001) observed increases in root formation of miniature rose cuttings after adding whole inoculum containing the arbuscular mycorrhizal fungus *Glomus intraradices*. Another study with the same host plant and inoculum type found not only improved rooting following the inoculations, but concentrations of some nutrients in the cuttings were also increased (Scagel, 2004). In both of these studies, the effects on plant rooting and nutrient uptake were observed while the roots were devoid of colonization.

Although it is widely accepted that arbuscular mycorrhizal fungi are obligate biotrophs that cannot be cultured outside of root systems (Williams, 1992), there have been studies demonstrating that some arbuscular mycorrhizal fungi can survive independently in soil for brief periods without colonizing roots (Giovannetti et al., 1996; Gadkar et al., 2001). Although not well-understood, there is evidence that some *Glomus* and *Gigaspora* species grow in response to biochemical signals, such as ethylene, exuded from the plants (Vierheilig et al., 1994). Plant roots also secrete a group of secondary metabolites called strigolactones that function as host recognition signals for arbuscular mycorrhizal fungi (Bécard et al., 2004; Yoneyama et al., 2007). Strigolactones, such as 5-deoxystrigol, have been shown to induce hyphal branching in arbuscular mycorrhizal fungi to prepare the mycobiont for symbiosis (Yoneyama et al., 2007). Thus, even in the absence of colonization, plants may

affect the biological activity of fungi in the soil, which in turn may affect plant development and nutrition. Plant responses to arbuscular mycorrhizal inoculum prior to colonization are thought to be associated with changes in plant flavonoid levels, as Larose et al. (2002) observed flavonoid accumulation in un-colonized *Medicago sativa* roots following inoculation with three arbuscular mycorrhizal fungi. However, the relationship between flavonoid accumulation trends and altered growth or nutrition has not been fully explored.

Similar results have been demonstrated in studies using ectomycorrhizal inoculum. *Wilcoxina mikolae* is capable of forming both ectendomycorrhizal and ectomycorrhizal associations (Yu et al., 2001), but can reside in the soil independently of plant roots and thus remain capable of altering rhizosphere dynamics. Levisohn (1956) reported improved seedling nutrition and growth, without root colonization, following inoculation of *Picea*, *Pinus*, and *Betula* species with the ectomycorrhizal fungi *Rhizopogon luteolus* and *Boletus scaber*. It was suggested that the plants were influenced by the release of nutrients from soil organic matter by the fungal mycelia (Levisohn, 1956). A similar study found that inoculating *Arctostaphylos uva-ursi* and *Vaccinium ovatum* cuttings with 13 ectomycorrhizal fungi was related to improved rooting percentages and root growth without the occurrence of colonization (Linderman and Call, 1977). These studies demonstrate that both arbuscular mycorrhizal and ectomycorrhizal fungi are capable of eliciting responses in host and non-host plants without forming a symbiosis; however, it is unclear if this is what transpired in the current study. In future work, a proper control treatment receiving autoclaved inoculum in place of sterile sand may account for nutrients added to the substrate via the inoculum (Koide and Li, 1989).

There are several other possibilties that may explain why tissue nutrient concentrations differed between treatments. Although not likely, mycorrhizal colonization may have been present but went undetected through the harvesting and analysis of the roots. Also, the endophytic fungal structures observed in the roots may have affected nutrient uptake of the host plants. This is difficult to assess in the current study because endophyte structures were present in all examined roots and were not compared between treatments. Although fungal endophytes are ubiquitous in nature, their effects on plant nutrient uptake are poorly understood and have not been examined for Wollemi Pines (Saito et al., 2006). In addition, microbes other than the intended fungi may have been introduced into the soil as part of the inoculum. Therefore, treating control plants with washings from inoculum may incorporate microbes present in the inoculum without adding the mycorrhizal fungi (Koide and Li, 1989). Despite the possible explanations for differences in treatment nutrient accumulation in the plant tissue, more research is needed to definitively explain these differences.

Although both experiments involved similar inoculation methods and shared some of the same mycorrhizal fungal species, the plant growth responses differed between the two experiments. More specifically, plants inoculated with the *Glomus* species exhibited a positive growth response as compared to the sterile sand treatment in the first experiment, while the growth of *G. clarum*-inoculated plants in the second experiment was significantly reduced as compared to the control plants. The positive growth trends in the first experiment may have been due to supplemented nutrients within the inoculum that were not accounted for, but that does not explain the growth reductions in the second experiment because a proper control treatment of uncolonized leeks was incorporated. Differences between the

two experiments include the growth substrate and the source of inoculum, both of which may have affected plant growth. The observed differences in growth without root colonization may also be related to the plant-fungus interactions within the substrate that were described in the previous paragraph. Further experimentation is required to better understand the differences in plant growth responses between the two studies.

2.5 Conclusion

This is the first study to investigate the effects of inoculating Wollemi Pines with mycorrhizal fungi. This work exemplifies the challenges in working with a rare species with relatively little background information, especially in terms of its mycorrhizal associations. Although mycorrhizal colonization was not observed in inoculated plants, this study provides a starting point in studying interactions of Wollemi Pine with mycorrhizal fungi. Further research is required to explain the differences in tissue nutrient concentration between trees exposed to inoculum and the control treatments. The endophytic fungal structures consistently observed in the roots are examined in Chapter 3. Studies described in Chapter 4 explore potential allelopathic effects of Wollemi Pines on leek germination and growth. A better understanding of this fascinating species will contribute to the success of its re-establishment in the wild.

2.6 References

Amijee F; Stribley DP; Tinker PB. (1990) Soluble carbohydrates in roots of leek (*Allium porrum*) plants in relation to phosphorus supply and VA mycorrhizas. Plant and Soil. 124: 195-198

Bécard G; Kosuta S; Tamasloukht M; Séjalon-Delmas N; Roux C. (2004) Partner communication in the arbuscular mycorrhizal interaction. Can. J. Bot. 82: 1186-1197

Blanke V; Renker C; Wagner M; Füllner K; Held M; Kuhn AJ; Buscot F. (2005) Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. New Phytologist. 166: 981-992.

Brundrett MC; Piché Y; Peterson RL. (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can. J. Bot. 62: 2128-2134

Brundrett MC; Jasper DA; Ashwath N. (1999) Glomalean mycorrhizal fungi from tropical Australia II. The effects of nutrient levels and host species on the isolation of the fungi. Mycorrhiza. 8: 315-321

Daniels BA; Skipper HD. (1982) Methods for the recovery and quantitative estimation of propagules from soil. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 29-35

de Miranda JCC; Harris PJ. (1994) Effects of soil phosphorus on spore germination and hyphal growth of arbuscular mycorrhizal fungi. New Phytologist. 128: 103-108

Duponnois R; Plenchette C. (2003) A mycorrhizal helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acacia* species. Mycorrhiza. 13: 85-91

Ferguson JJ; Woodhead SH. (1982) Production of endomycorrhizal inoculum. A. Increase and maintenance of vesicular-arbuscular mycorrhizal fungi. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 47-54

Gadkar V; David-Schwartz R; Kunik T; Kapulnik Y. (2001) Arbuscular mycorrhizal fungal colonization. Factors involved in host recognition. Plant Physiology. 127: 1493-1499

Geil RD; Guinel FC. (2002) Effects of elevated substrate-ethylene on colonization of leek (*Allium porrum*) by the arbuscular mycorrhizal fungus *Glomus aggregatum*. Can. J. Bot. 80: 114-119

Giovannetti M; Sbrana C; Citernesi AS; Avio L. (1996) Analysis of factors involved in fungal recognition responses to host-derived signals by arbuscular mycorrhizal fungi. New Phytologist. 133: 65-71

Hamel C; Dalpé Y; Furlan V; Parent S. (1997) Indigenous populations of arbuscular mycorrhizal fungi and soil aggregate stability are major determinants of leek (*Allium porrum* L.) response to inoculation with *Glomus intraradices* Schenck & Smith or *Glomus versiforme* (Karsten) Berch. Mycorrhiza. 7: 187-196

Hardie K; Leyton L. (1981) The influence of vesicular-arbuscular mycorrhiza on growth and water relations of red clover. I. In phosphate deficient soil. New Phytologist. 89: 599-608

Horwitz W. (2000) Official methods of analysis of AOAC international, 17th Ed. Gaithersburg, Maryland: Association of Official Analytical Chemists

Johansen A; Jakobsen I; Jensen ES. (1994) Hyphal N transport by a vesicular-arbuscular mycorrhizal fungus associated with cucumber grown at three nitrogen levels. Plant and Soil. 160: 1-9

Jones WG; Hill KD; Allen JM. (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. Telopea. 6: 173-176

Klironomos JN. (2003) Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology. 84: 2292-2301

Koide RT; Li M. (1989) Appropriate controls for vesicular-arbuscular mycorrhiza research. New Phytologist. 111: 35-44

Larose G; Chênevert R; Moutoglis P; Gagné S; Piché Y, Vierheilig H. (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. Journal of Plant Physiology. 159: 1329-1339

Lesica P; Antibus RK. (1990) The occurrence of mycorrhizae in vascular epiphytes of two Costa Rican rain forests. Biotropica. 22: 250-258

Levisohn I. (1956) Growth stimulation of forest tree seedlings by the activity of free-living mycorrhizal mycelia. Forestry. 29: 53-59

Linderman RG; Call CA. (1977) Enhanced rooting of woody plant cuttings by mycorrhizal fungi. Journal of the American Society for Horticultural Science. 102: 629-632

Marx DH. (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology. 59: 153-163

Marx DH; Kenney DS. (1982) Production of ectomycorrhizal fungus inoculum. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 131-146

McGee PA; Bullock S; Summerell BA. (1999) Structure of mycorrhizae of the Wollemi pine (*Wollemia nobilis*) and related Araucariaceae. Aust. J. Bot. 47: 85-95

McGonigle TP; Miller MH; Evans DG; Fairchild GL; Swan JA. (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist. 115: 495-501

Menge JA, Timmer LW. (1982) Procedure for inoculation of plants with vesicular-arbuscular mycorrhizae in the laboratory, greenhouse, and field. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 59-68

Molina R; Palmer JG. (1982) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 115-130

Morrison TM; English DA. (1967) The significance of mycorrhizal nodules on *Agathis australis*. New Phytologist. 66: 245-250

Newsham KK; Fitter AH; Watkinson AR. (1994) Root pathogenic and arbuscular mycorrhizal fungi determine fecundity of asymptomatic plants in the field. Journal of Ecology. 82: 805-814

Newsham KK; Fitter AH; Watkinson AR. (1995) Multi-functionality and biodiversity in arbuscular mycorrhizas. TREE. 10: 407-411

Peat HJ; Fitter AH. (1993) The distribution of arbuscular mycorrhizas in the British flora. New Phytologist. 125: 845-854

Peterson RL; Massicotte HB; Melville LH. (2004) Mycorrhizas: anatomy and cell biology. Ottawa, Ontario: NRC Research Press.

Saito K; Kuga-Uetake Y; Saito M; Peterson RL. (2006) Vacuolar localization of phosphorus in hyphae of *Phialocephala fortinii*, a dark septate fungal root endophyte. Can. J. Microbiol. 52: 643-650

Sanders IR. (2003) Preference, specificity and cheating in the arbuscular mycorrhizal symbiosis. Trends in Plant Science. 8: 143-145

Scagel CF. (2001) Cultivar specific effects of mycorrhizal fungi on the rooting of miniature rose cuttings. Journal of Environmental Horticulture. 19: 15-20

Scagel CF. (2004) Changes in cutting composition during early stages of adventitious rooting of miniature rose altered by inoculation with arbuscular mycorrhizal fungi. Journal of the American Society for Horticultural Science. 129: 624-634

Singh HP; Kohli RK; Batish DR; Kaushal PS. (1999) Allelopathy of gymnospermous trees. Journal of Forest Research. 4: 245-254

Smith SE; Read DJ. (1997) Mycorrhizal symbiosis. 2nd Ed. San Diego, California: Academic Press, Inc.

Vierheilig H; Alt M; Mohr U; Boller T; Wiemken A. (1994) Ethylene biosynthesis and activities of chitinase and β-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. Journal of Plant Physiology. 143: 337-343

Vimard B; St-Arnaud M; Furlan V; Fortin JA. (1999) Colonization potential of in vitro-produced arbuscular mycorrhizal fungus spores compared with a root-segment inoculum from open pot culture. Mycorrhiza. 8: 335-338

White JA; Charvat I. (1999) The mycorrhizal status of an emergent aquatic, *Lythrum salicaria* L., at different levels of phosphorus availability. Mycorrhiza. 9: 191-197

Williams PG. (1992) Axenic culture of arbuscular mycorrhizal fungi. Methods in Microbiology. 24: 203-220

Wu T; Hao W; Lin X; Shi Y. (2002) Screening of arbuscular mycorrhizal fungi for the revegetation of eroded red soils in subtropical China. Plant and Soil. 239: 225-235

Yang CS; Korf RP. (1985) A monograph of the genus *Tricharina* and of a new, segregate genus, *Wilcoxina* (Pezizales). Mycotaxon. 24: 467-531

Yoneyama K; Xie X; Kusumoto D; Sekimoto H; Sugimoto Y; Takeuchi Y; Yoneyama K. (2007) Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. Planta. 227: 125-132

Yu TEJ-C; Egger KN; Peterson RL. (2001) Ectendomycorrhizal associations – characteristics and functions. Mycorrhiza. 11: 167-177

Zandavalli RB; Dillenburg LR; de Souza PVD. (2004) Growth response of *Araucaria angustifolia* (Araucariaceae) to inoculation with the mycorrhizal fungus *Glomus clarum*. Applied Soil Ecology. 25: 245-255

Chapter 3: Field Inoculation of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) with Arbuscular Mycorrhizal Fungi[2]

3.1 Introduction

A new plant taxon, *Wollemia nobilis* Jones, Hill & Allen (Wollemi Pine), was recently discovered in a rainforest gorge in Wollemi National Park, Australia (Jones et al., 1995). The Wollemi Pine is highly endangered, with less than 100 mature trees at three remote sites within the park (Offord and Meagher, 2006). As a new member of the ancient conifer family Araucariaceae, the Wollemi Pine has attracted international attention for both its rarity and uniqueness as a novel monotypic genus. The trees are easily propagated from seeds and cuttings (Bardell, 2006), providing researchers with an opportunity to explore different properties of a living fossil.

The world's Wollemi Pine populations have been naturally reduced over time by climate and geographical changes, but now human-induced dangers are the focus of conservation research. The current population has remarkably low genetic diversity (Peakall et al., 2003), making the entire species extremely vulnerable to extinction. Pathogens pose a significant threat to the population, as Wollemi Pines have exhibited susceptibility to pathogenic microbes that could easily be introduced into their natural habitat (Bullock et al., 2000). Thus, plans for Wollemi Pine conservation efforts include out-planting laboratory-propagated seedlings to establish larger and less vulnerable populations. However, surviving

[2] A version of this chapter will be submitted for publication. Biggs LE; Murch SJ; Durall DM. Field Inoculations of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) with Arbuscular Mycorrhizal Fungi.

the initial establishment period will be challenging for the trees since their natural habitat consists of sandstone-derived soils that are notably low in mineral nutrients and water-retaining capacity (Beadle, 1962).

Mycorrhizal fungi can access mineral nutrients that are physically or biochemically unavailable to plants and supply them to their host in exchange for photosynthate (Smith and Read, 1997). Thousands of mycorrhizal fungal species form mutualistic symbioses with the roots of over 90 % of terrestrial plants (Peterson et al., 2004). To date, one study has inspected Wollemi Pine roots from the native population for mycorrhizal associations (McGee et al., 1999), and found colonization by arbuscular mycorrhizal fungi and *Wilcoxina*-like fungi. However, more research is required to determine the effects of these associations on the survival, growth, and nutrient acquisition of Wollemi Pines in a forest setting.

The overall objective of this study was to determine the ability of native Okanagan and commercially-supplied arbuscular mycorrhizal fungi to colonize the roots of Wollemi Pines planted into a ponderosa pine forest. The specific objectives were (a) to identify mycorrhizal and endophytic fungi that had colonized roots of Wollemi Pine in the ponderosa pine forest, (b) to determine whether *Glomus intraradices* applied as commercial inoculum or as colonized leek root fragments at the time of planting would colonize the young Wollemi Pines, (c) to identify the arbuscular mycorrhizal fungi colonizing native grasses in the ponderosa pine forest and compare them to those in roots of planted Wollemi Pine, and (d) to determine the effects of different environmental conditions and fungal treatments on the nutrient content of field-grown Wollemi Pines.

3.2 Materials and Methods

3.2.1 Site Characteristics

The Wollemi Pines were planted near Kelowna in the southern interior of British Columbia (49 ^{o}59 'N, 119 ^{o}26 'W) (Figure 3.1). The study area was approximately 30 m by 60 m and ranged in elevation from 386 m to 403 m. The trees were planted on a north-facing slope bordering a ravine. Throughout the site, ponderosa pine (*Pinus ponderosa* Dougl.) was a dominant overstorey species, along with minor components of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). Common understorey plants included arrow-leaf balsamroot (*Balsamorhiza sagittata* (Pursh.) Nutt), Oregon grape (*Mahonia aquifolium* (Pursh.) Nutt), and bluebunch wheatgrass (*Pseudoroegneria spicata* (Pursh.) A. Löve). According to the British Columbia Ministry of Forests, the study area is part of the ponderosa pine biogeoclimatic zone due to its warm climate, low elevation, and dominant vegetation (Lloyd et al., 1990). In this climate, mean minimum and maximum temperatures range from 0 ^{o}C/-7 ^{o}C in January to 13 ^{o}C/28 ^{o}C in July (Lloyd et al., 1990). The average annual rainfall in the ponderosa pine biogeoclimatic zone is approximately 280 mm (Lloyd et al., 1990).

Figure 3.1 Aerial image depicting the location near Kelowna, British Columbia where the Wollemi Pines were planted in May 2008 (49 °59 'N, 119 °26 'W). Note that the planting area (indicated by the white arrow) was adjacent to a ravine and 60 m uphill from the shores of Okanagan Lake.

3.2.2 Experimental Design and Planting

The Wollemi Pines used in this study were propagated from cuttings in Australia and entered Canada in 2005 in accordance with CFIA permits, inspections, and regulations. The plants were three years old when transferred from Nordic Nurseries in Abbotsford, British Columbia to UBC Okanagan in Kelowna, British Columbia in May 2008. Upon arrival at UBC Okanagan, the shoot height of the plants ranged from 30 to 50 cm and they were

growing in peat-based substrate in 15 cm diameter pots. The trees were immediately planted in the study area.

Fifty-six trees were planted according to the following three treatments: local soil without additional mycorrhizal inoculum, local soil with leek root inoculum, and potting mix containing commercial inoculum (Figures 3.2 and 3.3). The planting spots for all experimental trees were selected to be close to potential inoculum sources, particularly bluebunch wheatgrass roots. All of the Wollemi Pines were planted at least 1 m apart. After removing the plants from the pots, the previous substrate was left undisturbed to protect the root balls. The holes were dug 20 cm deep and 20 cm in diameter, which was as small as possible to minimize disturbance to the surrounding soil and potential inoculum sources. The inocula were mixed with the soil that had been removed from the planting holes, which was then used to plant the trees. Thirty-two of the trees were planted using only local soil without additional fungal inoculum, while 16 trees were inoculated with leek (*Allium porrum* L. cv. Giant Musselburgh) roots heavily colonized by *Glomus intraradices* Schenck & Smith (section 2.2.3.1; Figure 3.4). Ten grams of 2 to 3 cm long leek root fragments were mixed with the soil used to plant each tree (Menge and Timmer, 1982). The third treatment consisted of eight trees planted using 2 L of Premier PRO-MIX® BX commercial growth medium (Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada) per plant instead of local soil. The growth medium contained 1 *Glomus intraradices* fungal propagule per gram, as indicated by the label. These eight trees were also supplemented with 4 L of 100 % natural cedar bark mulch (Nu-Gro Inc, Brantford, Ontario, Canada) on the soil surface.

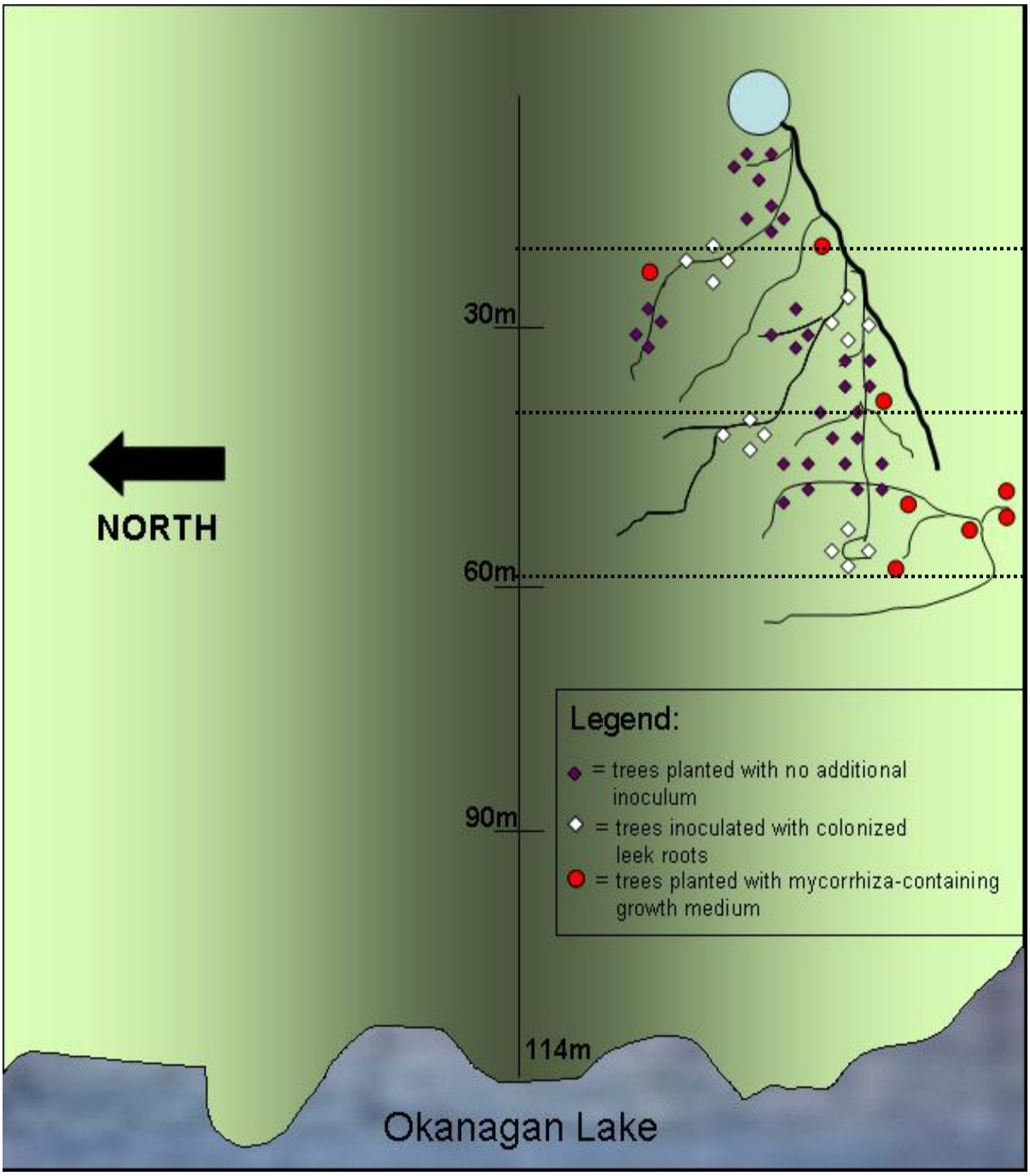

Figure 3.2 Schematic map of Wollemi Pine planting locations. Distances in metres from the 500 gallon water tank are indicated on the figure. Dashed horizontal lines indicate regions from which soil samples were taken.

Figure 3.3 *Wollemia nobilis* trees planted at the study area near Kelowna, British Columbia (indicated by the grey arrows). Note that the plants were watered using gravity-fed drip irrigation (indicated by the black arrow).

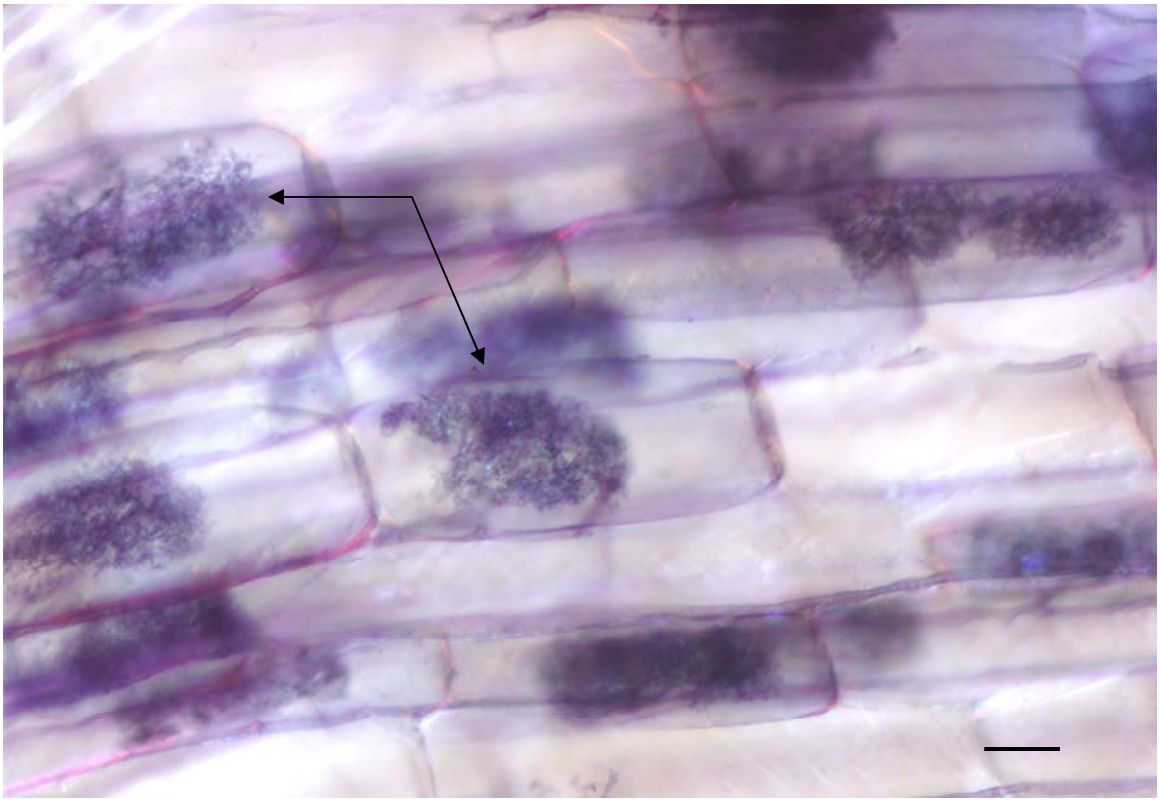

Figure 3.4 Leek root stained with chlorazol black E and viewed under the 20X objective of a light microscope (Bar = 20 μm), used as an inoculum source for Wollemi Pines. Note the intracellular arbuscules formed by the fungus *Glomus intraradices* (indicated by the arrows).

3.2.3 Growth and Monitoring of Seedlings

Plant heights from the soil surface to the top of the main growing stem were measured on a monthly basis. The plants were watered as needed using a gravity-fed drip irrigation system. Briefly, water was delivered to each of the plants from a 2000 L water tank through a series of 1.25 cm diameter lines and polyethylene micro-tubing, and discharged from emitters at the base of each plant. The irrigation system was manually controlled and the frequency of watering was weather-dependent. For example, the plants

were watered for at least one hour every second day during hot and dry weather. The irrigation system was regularly inspected for leaks, clogs, or shifted lines.

Soil samples were taken from the site in July 2008. To obtain samples that were representative of the planting area, the site was divided into three regions: the uppermost third of the slope (0 to 20 m downslope from the water tank), the middle region of the slope (20 to 40 m downslope from the water tank), and the bottom third of the slope (40 to 60 m downslope from the water tank) (Figure 3.2). Two soil samples were taken from each region in close proximity to out-planted Wollemi Pines, and the six samples were sent to CANTEST Ltd (Burnaby, B.C., Canada) for analysis of moisture, pH, organic matter, total carbon, and available nitrogen, phosphorus, potassium, calcium, and magnesium by a flow injection analyzer (N), Inductively Coupled Argon Plasma (ICAP) Spectroscopy (cations), and combustion in an induction furnace (organic matter, total C) (Carter, 1993). Moisture was quantified gravimetrically and pH was determined after leaching with deionized water.

Photosynthetically active radiation (PAR) received by the plants was measured twice on a clear sunny day, once in mid-morning and once in early afternoon in August 2008. Measurements were taken using a LI-COR LI-250A Light Meter (LI-COR Biotechnology, Lincoln, Nebraska, U.S.A.) at plant height for all 56 plants. Lastly, an Onset Weather Station was installed at the planting site to measure temperature, rainfall, relative humidity, and soil water content at 15 minute intervals throughout the latter three months of the study (Hoskin Scientific Ltd., Vancouver, B.C., Canada) (Figure 3.5). Volumetric soil water content was measured at one location using an ECH_2O Probe (Decagon Devices Inc, Pullman, Washington, U.S.A.) situated alongside Wollemi Pines planted in the lower region of the site.

Figure 3.5 Weather station installed at the Wollemi Pine planting area to log information about local temperature, rainfall, relative humidity, and soil moisture.

3.2.4 Sampling of Bluebunch Wheatgrass Roots, Observation of Fungal Root Colonization, and Identification of Fungal Symbionts

Bluebunch wheatgrass was a dominant understorey plant and surrounded each experimental plant, thus making it the most likely inoculum source for the Wollemi Pine roots. To confirm that the grass roots were colonized with mycorrhizal fungi, root samples from 24 bluebunch wheatgrass plants throughout the study area were taken in July 2008 and examined for colonization. The samples were randomly collected throughout the site but all were in close proximity to outplanted Wollemi Pines. After collection, the roots were gently

washed to remove soil and were stored at -20 ^{o}C for one week prior to analysis. Approximately fifteen 2 to 3 cm root fragments were randomly selected from each grass sample for microscopic analysis. The grass roots were cleared and stained using the chlorazol black E method (Brundrett et al., 1984), with some slight modifications due to the delicate nature of the roots. Briefly, the roots were placed in staining baskets lined with nylon mesh, immersed in 10 % potassium hydroxide, and autoclaved for five minutes on the slow exhaust cycle. After rinsing with several changes of water, the baskets were heated in chlorazol black E staining solution (consisting of equal parts of 0.1 % (w/v) chlorazol black E solution, 85 % lactic acid, and glycerol) at 90 ^{o}C for one hour. The baskets were then submerged in glycerol overnight to destain the roots. The following day, the roots were viewed and photographed using a Nikon Eclipse E800 light microscope, a Nikon DXM1200 digital camera, and Nikon Act-1 Version 2.20 software (LEAD Technologies, Inc). Fungal colonization of the roots was quantified using the magnified intersections method of McGonigle et al. (1990). In brief, microscope slides of stained roots were inspected by six vertical passes using an ocular lens with vertical and horizontal crosshairs. Where the center of the crosshairs intersected a root, an observation was made as to the presence or absence of arbuscular mycorrhizal fungal structures (arbuscules, vesicles, or hyphae). The number of intersection points containing fungal structures was divided by the total number of intersection points to provide a percent colonization value.

Molecular methods were used to identify fungi colonizing the bluebunch wheatgrass roots. The roots were stored at -20 ^{o}C for approximately six weeks prior to DNA extraction. For each of the 24 bluebunch wheatgrass plants, a 1 cm root fragment was randomly sampled and ground with a ceramic bead using a Thermo Savant FastPrep FP120 grinder (Qbiogene

Inc, Carlsbad, California, U.S.A.). Genomic DNA was extracted from the ground samples using a Qiagen DNeasy Plant Tissue Kit (Qiagen Inc, Mississauga, Ontario, Canada) as per the manufacturer's instructions. To target the major fungal phyla that form mycorrhizas, each extracted sample was subjected to two different PCR reactions using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, U.S.A.). Components of the PCR reaction mixtures for each primer pair are found in Table 3.1. The NS31/AM1 primer pair was used to target a 550 bp region of the small subunit ribosomal DNA (SSU) of arbuscular mycorrhizal fungi belonging to the Glomeromycota (Simon et al., 1992; Helgason et al., 1998). Although the NS31/AM1 primer pair reportedly omits two clades of arbuscular mycorrhizal fungi (Paraglomaceae and Archeosporaceae) (Schüβler et al., 2001), these primers have been used in several recent field studies to amplify fungal DNA sequences from roots (e.g. Vandenkoornhuyse et al., 2003). The reaction was heated at 94 ^{o}C for 10 minutes, followed by 35 cycles of 45 seconds at 94 ^{o}C, one minute at 58 ^{o}C, and one minute at 72 ^{o}C, and followed by a seven minute extension period at 72 ^{o}C. The second primer pair used in PCR was ITS1/ITS4, which targets an 800 bp region of the internal transcribed spacer (ITS) regions of fungal ribosomal DNA for the Ascomycota and Basidiomycota phyla, containing fungi that form ectomycorrhizas and ectendomycorrhizas (Gardes and Bruns, 1993; Martin and Rygiewicz, 2005). The non-coding but highly variable ITS1 and ITS2 regions that are amplified with the ITS1/ITS4 primer pair exhibit known species-specific polymorphisms that permit accurate identification of many fungi (e.g. Kovács et al., 2001). After a 10 minute initial denaturation step at 95 ^{o}C, the temperature profile was programmed to repeat 35 cycles of 45 seconds at 94 ^{o}C, 45 seconds at 55 ^{o}C, and one minute at 72 ^{o}C, followed by a 10 minute extension period at 72 ^{o}C. Products of the PCR reactions were separated on 1 %

agarose gels using SYBR Safe® DNA stain (Invitrogen Life Technologies, Carlsbad, California, U.S.A.) and the gels were visualized under UV illumination using a KODAK Gel Logic 200 Imaging System (Kodak Company, Rochester, New York, U.S.A.). Excess primers and nucleotides were removed from the amplified samples using ExoSAP-IT™ reagent (USB Corporation, Cleveland, Ohio, U.S.A.), as per the manufacturer's instructions. Following the clean-up step, sequencing was performed using the Big Dye Terminator Kit on a 3130 x l capillary sequencer (Applied Biosystems, Foster City, California, U.S.A.). Forward and reverse sequences were aligned and manually corrected using Sequencher 4.6 software (GeneCodes, Ann Arbor, Michigan, U.S.A.). Poor quality sequences that did not align properly were analyzed only in the direction of highest quality. Sequences were entered into the Basic Local Alignment Search Tool (BLAST) search program (Altschul et al., 1997) to determine taxonomic matches for each sample using the National Center for Biotechnology Information (NCBI) nucleotide database. Sequence similarities of 98 % or greater were considered to indicate the same molecular species or operational taxonomic unit. For matches with unknown fungal cultures, the sequences were aligned with those of fifteen randomly selected *Glomus* spp. voucher specimens from the NCBI nucleotide database using the default settings of the T-Coffee program (European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom). The resulting alignment data was edited using Bio Edit Sequence Alignment Editor 7.0.9.0 (Ibis Biosciences, Carlsbad, California, U.S.A.) and a phylogenetic tree was constructed with MEGA4 (Biodesign Institute, Tempe, Arizona, U.S.A.).

Table 3.1 PCR reaction contents using template DNA extracted from bluebunch wheatgrass roots. Note that reaction volumes were made up to 25.00 µL using sterile water.

Reaction component	NS31/AM1	ITS1/ITS4
Reaction buffer (µL)	2.50	2.50
1 % BSA (bovine serum albumin)(µL)	1.00	2.00
10 mM dNTP blend (µL)	2.00	0.50
100 mM $MgCl_2$ (µL)	1.50	2.50
10 µM forward primer (µL)	0.60	1.00
10 µM reverse primer (µL)	0.60	1.00
5 U/mL *Taq* polymerase (µL)	0.20	0.20
Template DNA (µL)	1.00	3.00

3.2.5 Harvesting of Wollemi Pines and Observation of Fungal Root Colonization

In September 2008, four months after planting, all of the Wollemi Pines were harvested for analysis. The plants were separated at the root crown and the shoot portions were put into paper bags and weighed to determine wet tissue weights. The bags were oven-dried at 45 ^{o}C to a constant weight. The dried shoot tissue (leaves and stems) was re-weighed and ground to pass a 1 mm sieve using a Retsch® SM2000 grinding mill (Retsch Inc, Newtown, Pennsylvania, U.S.A.). Ground shoot samples from eight randomly selected trees of each of the three treatments were sent to CANTEST Ltd in Burnaby, British

Columbia for analysis of the following components: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, protein, nitrogen, fat, moisture, ash, carbohydrates, and energy in units of calories and Joules. To measure mineral content in the tissue, the samples were digested using a nitric acid-hydrogen peroxide digestion procedure and analyses were performed using Inductively Coupled Argon Plasma Spectroscopy (ICP). Proximate analyses were performed as described by Horwitz (2000).

Following removal from the field, the roots of each plant were gently washed with tapwater and stored at -20 ^{o}C for two weeks prior to analysis. Sixty 2 to 3 cm root fragments from the outer root ball of each of the 56 harvested plants were cleared and stained using the chlorazol black E staining method (Brundrett et al., 1984), as described in section 3.2.3 but with some slight modifications. Briefly, the roots were put into staining baskets and autoclaved in 10 % potassium hydroxide for 15 minutes on the slow exhaust cycle. The roots were then bleached in 30 % hydrogen peroxide for 25 minutes, rinsed with several changes of water, and treated with 1 M hydrochloric acid for 15 minutes. The baskets were heated in chlorozal black E stain as previously described. After destaining overnight in glycerol, all 60 of the stained roots from each plant were viewed, photographed, and percent colonization was assessed (McGonigle et al., 1990). A 1 cm root sample was randomly taken from each plant for use in molecular identification work. Genomic DNA was extracted from each sample and subjected to two PCR reactions using the same primer pairs and reaction conditions as described in the previous section. The PCR products were visualized, purified, and sequenced as in section 3.2.3.

3.2.6 Data Analysis

Total growth increment for each plant was calculated as the difference between the initial height and the height immediately before harvesting. Individual shoot extension rates were quantified by calculating the average monthly height increases over the experimental period. Mean height increment and extension rates were calculated for each treatment.

Treatment differences in Wollemi Pine growth increment, extension rate, and shoot nutrient composition were evaluated by one-way analysis of variance (ANOVA) using the Generalized Linear Model (GLM) Procedure (SAS software; SAS Institute Inc, Cary, North Carolina, U.S.A.). Significant differences in treatment means with ANOVA were assessed using Duncan's Multiple Range test (DMRT). This analysis was performed using the shoot tissue data from eight randomly selected plants of each treatment (n = 24). The same procedure was also used to compare the composition of soil samples collected from the three different zones of the study area (n = 6) and to test for significant treatment differences in PAR received by the trees in both mid-morning (n = 56) and early afternoon (n = 56).

3.3 Results

3.3.1 Weather and Soil Conditions Over the Study Period

The weather station installed at the study site provided data on climate conditions and soil moisture for the latter three months of the study period. Rainfall varied from 1.2 mm to 19.4 mm per month, and total rainfall in July, August, and September 2008 was 23.6 mm (Figure 3.6). The temperature at the study site ranged from 5.7 to 40.3 ^{o}C, and the average

temperature over the three month period was 18.7 ^{o}C (Figure 3.7). The average relative humidity at the study site was 51.4 %, and the maximum and minimum relative humidity values were 92.9 % and 12.4 %, respectively (Figure 3.8). The soil water content remained close to zero for the majority of the study period, but did increase during a rainy period in August 2008 (Figure 3.9). Trees planted with commercial inoculum received significantly less PAR than the other treatments in mid-morning (P = 0.038, F = 3.57, df = 55) (Figure 3.10). In early afternoon, plants inoculated with colonized leek roots received significantly less PAR than the other treatments (P = 0.046, F = 3.29, df = 55) (Figure 3.11). Comparative analysis of soil nutritional parameters revealed that there were no statistically significant differences in pH, total carbon, organic matter, or mineral content (all P > 0.05) (Table 3.2). However, soil moisture content was significantly greater in the lower slope region of the study site than in the upper and middle areas of the slope (P = 0.022, F = 17.50, df = 5).

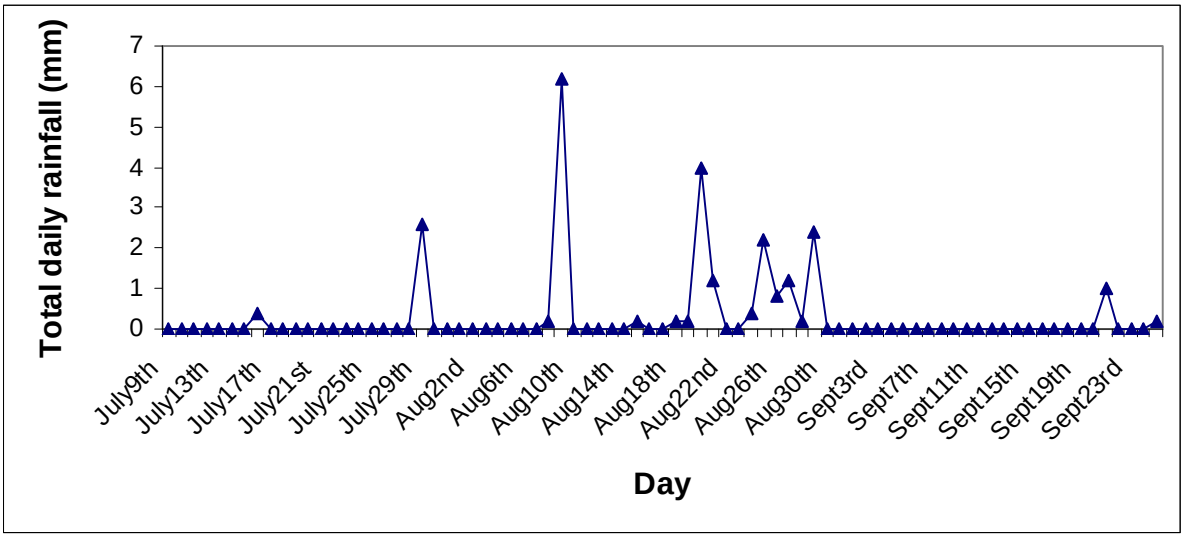

Figure 3.6 Rainfall patterns at the Wollemi Pine study area over the summer of 2008.

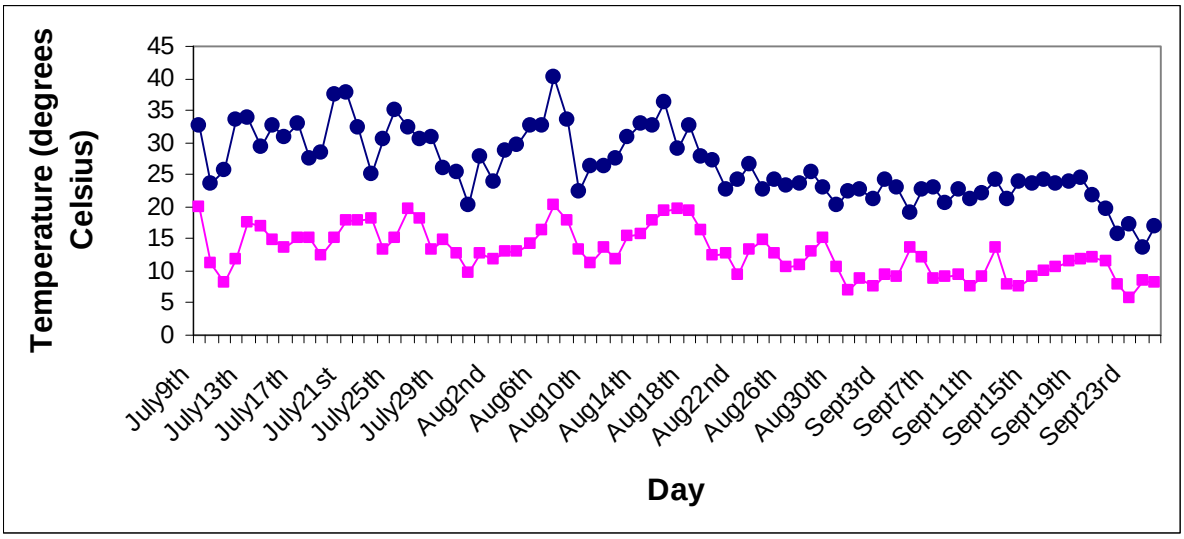

Figure 3.7 Daily minimum and maximum temperatures at the Wollemi Pine study area for the duration of the experiment. Circles = maximum daily values, squares = minimum daily values.

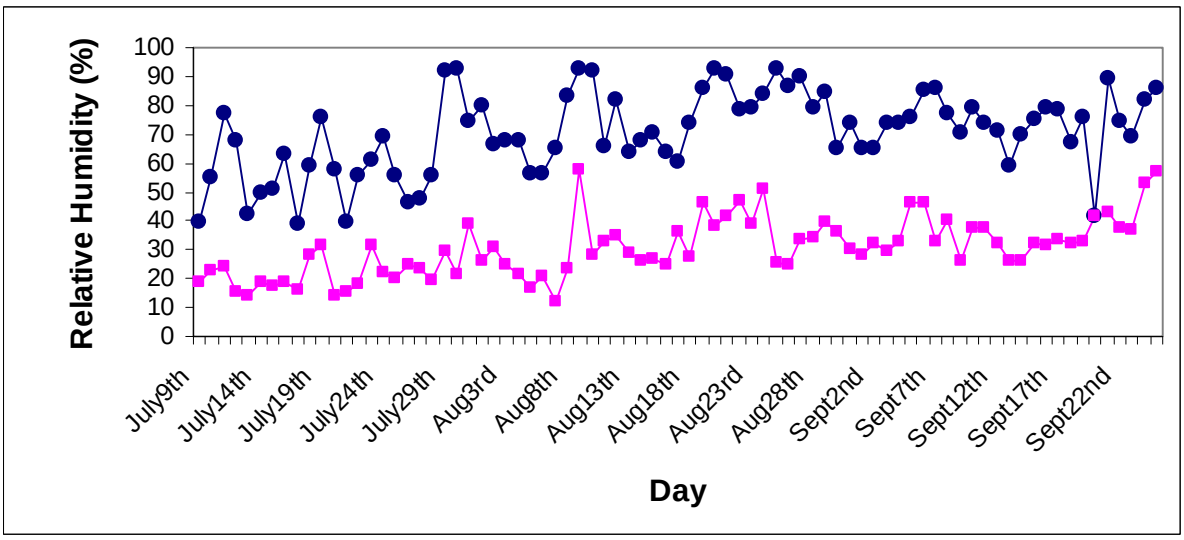

Figure 3.8 Daily minimum and maximum relative humidity measurements at the Wollemi Pine study area for the duration of the experiment. Circles = maximum daily values, squares = minimum daily values.

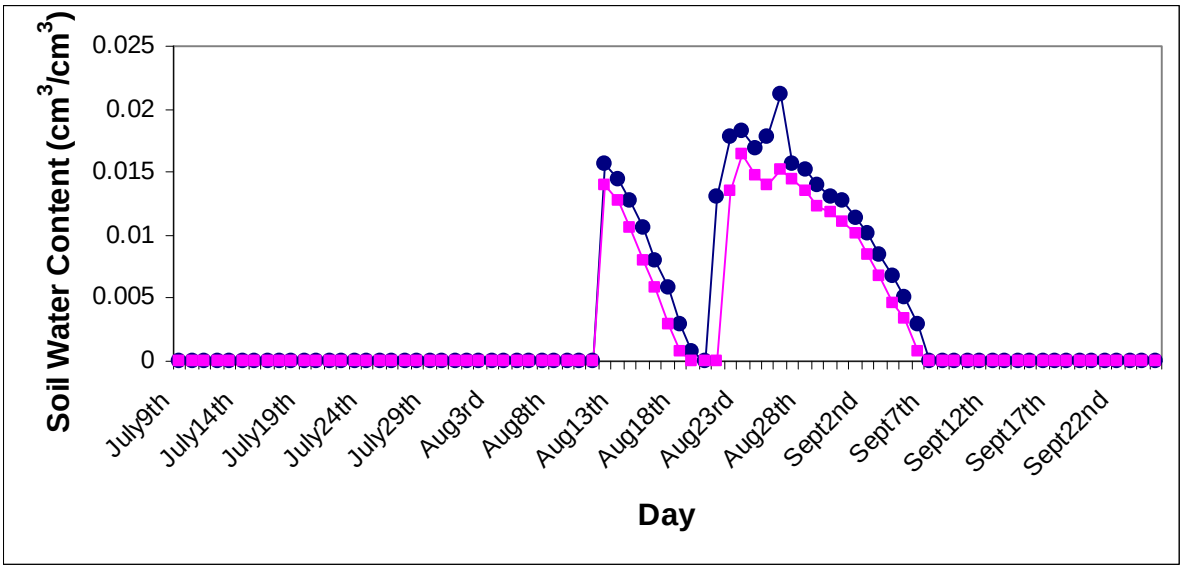

Figure 3.9 Daily minimum and maximum soil moisture content at the Wollemi Pine study area for the duration of the experiment. Circles = maximum daily values, squares = minimum daily values.

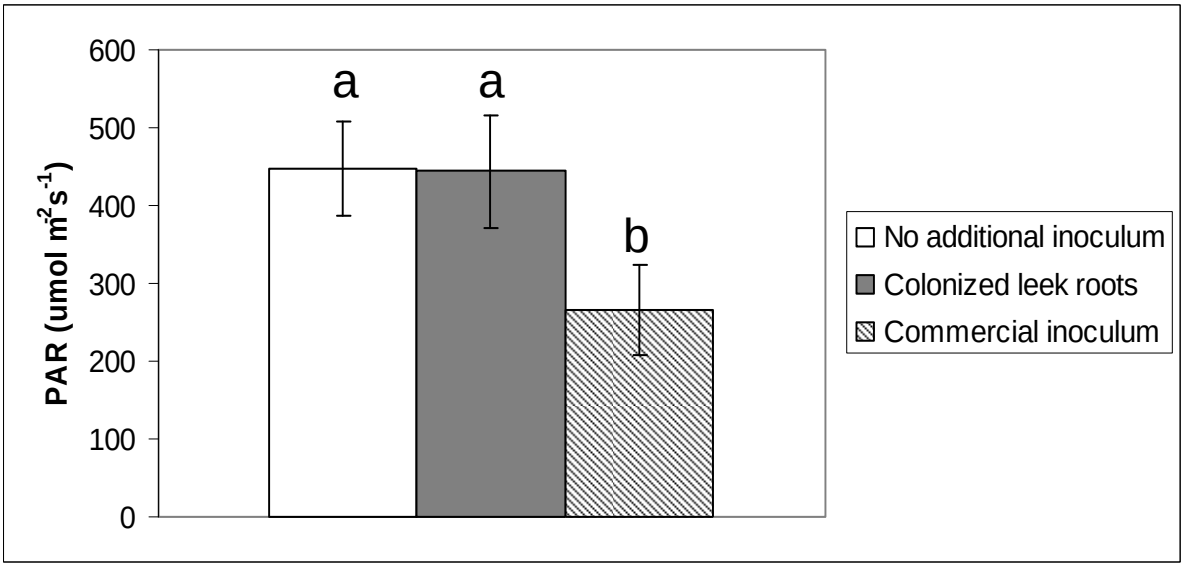

Figure 3.10 Photosynthetically active light received by all outplanted Wollemi Pines in mid-morning. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

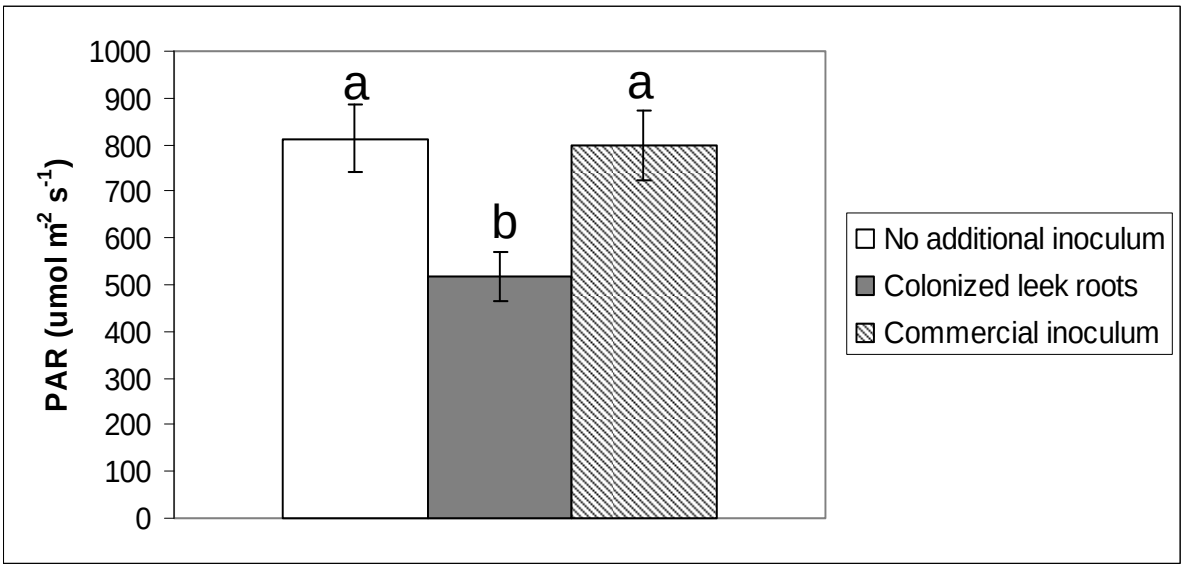

Figure 3.11 Photosynthetically active light received by all outplanted Wollemi Pines in early afternoon. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

Table 3.2 Mean nutritional parameters of soil samples taken from the three regions of the Wollemi Pine study area. Moisture content was the only parameter that significantly differed throughout the site (values with different superscripts are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test).

Soil parameter	Upper slope (mean +/- std error)	Mid-slope (mean +/- std error)	Lower slope (mean +/- std error)
Moisture (%)	$1.65 +/- 0.15^{b}$	$1.40 +/- 0.00^{b}$	$2.15 +/- 0.05^{a}$
Total C (%)	1.86 +/- 0.27	1.39 +/- 0.01	1.84 +/- 0.21
pH	6.50 +/- 0.00	6.90 +/- 0.30	6.70 +/- 0.00
Avail. N as Nitrate/Nitrite(μg/g)	1.00 +/- 0.40	0.68 +/- 0.18	0.50 +/- 0.00
Avail. N as Ammonia (μg/g)	2.57 +/- 0.03	1.69 +/- 0.41	1.63 +/- 0.66
Organic Matter (%)	3.50 +/- 0.50	2.00 +/- 0.00	3.50 +/- 0.50
Avail. P (μg/g)	41.00 +/- 5.00	38.50 +/- 5.00	32.00 +/- 1.00
Avail. Ca (μg/g)	1420 +/- 50	1410 +/- 80	1425 +/- 145
Avail. K (μg/g)	205 +/- 68	250 +/- 87	191 +/- 10
Avail. Mg (μg/g)	170 +/- 4	133 +/- 18	174 +/- 19

3.3.2 Fungal Endophytes of Wollemi Pines

Of the 56 Wollemi Pines harvested from the study area, mycorrhizal colonization was not observed in any of the roots that were stained and viewed with light microscopy. Therefore, the percentage of mycorrhizal colonization in the 60 observed roots was 0 % for each plant. However, all examined roots contained endophytic fungal structures, including networks of intercellular septate hyphae and intracellular hyphal coils (Figure 3.12).

Molecular analysis confirmed the presence of endophytic fungi. The NS31/AM1 primer pair produced very few, weakly-amplified products from Wollemi Pine roots. The ITS1/ITS4 primer pair generated the most consistent amplification of fungal DNA from the Wollemi Pine root fragments, producing products of at least 600 bp in length for 24 of 56 samples (Figure 3.13). Of the 24 samples that were sequenced, 14 produced short sequences of poor quality and 10 produced high quality sequences that were entered into the BLAST search. The taxa sharing the highest sequence similarity with the fungal DNA extracted from Wollemi Pine roots were *Cylindrocarpon pauciseptatum*, *Cylindrocarpon* sp., and *Phialocephala fortinii* (Table 3.3).

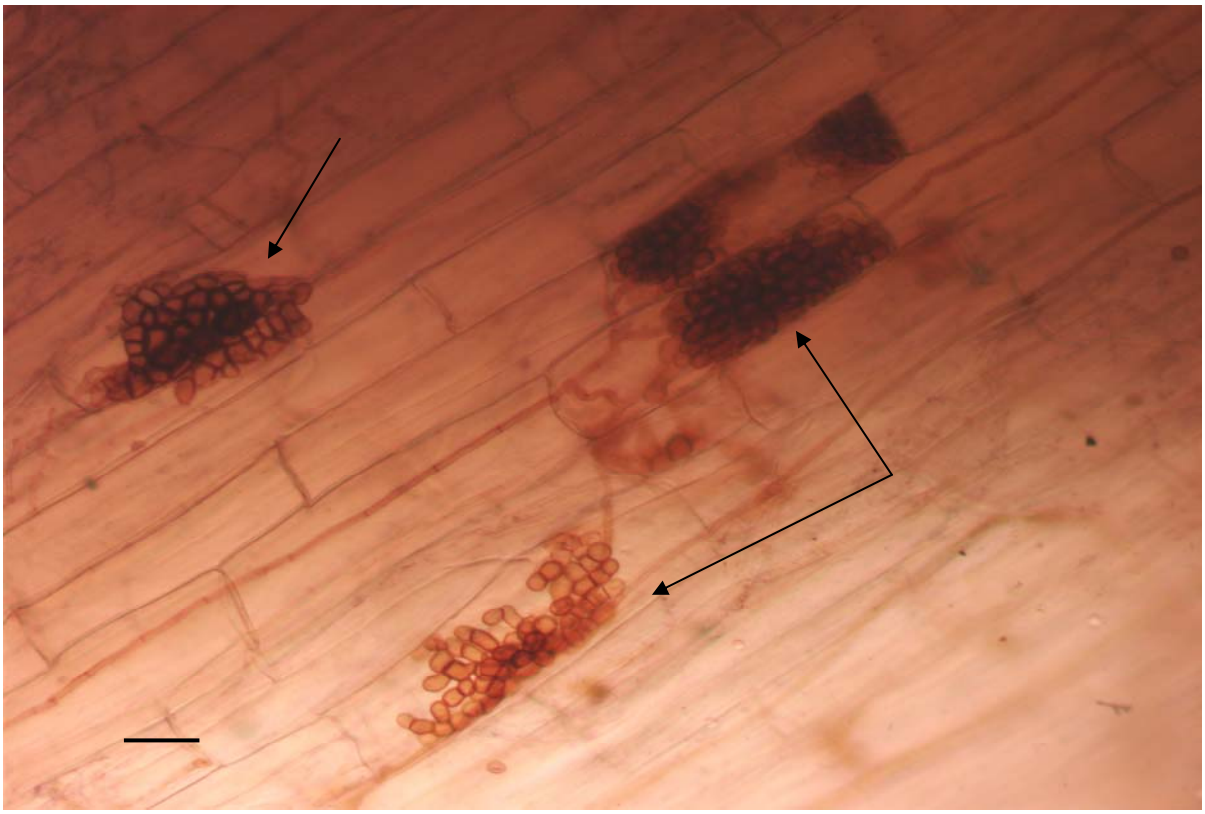

Figure 3.12 *Wollemia nobilis* root stained with chlorazol black E and viewed at 20X magnification (Bar = 20 µm). Note the intracellular hyphal aggregations of the endophytic fungus (indicated by the arrows).

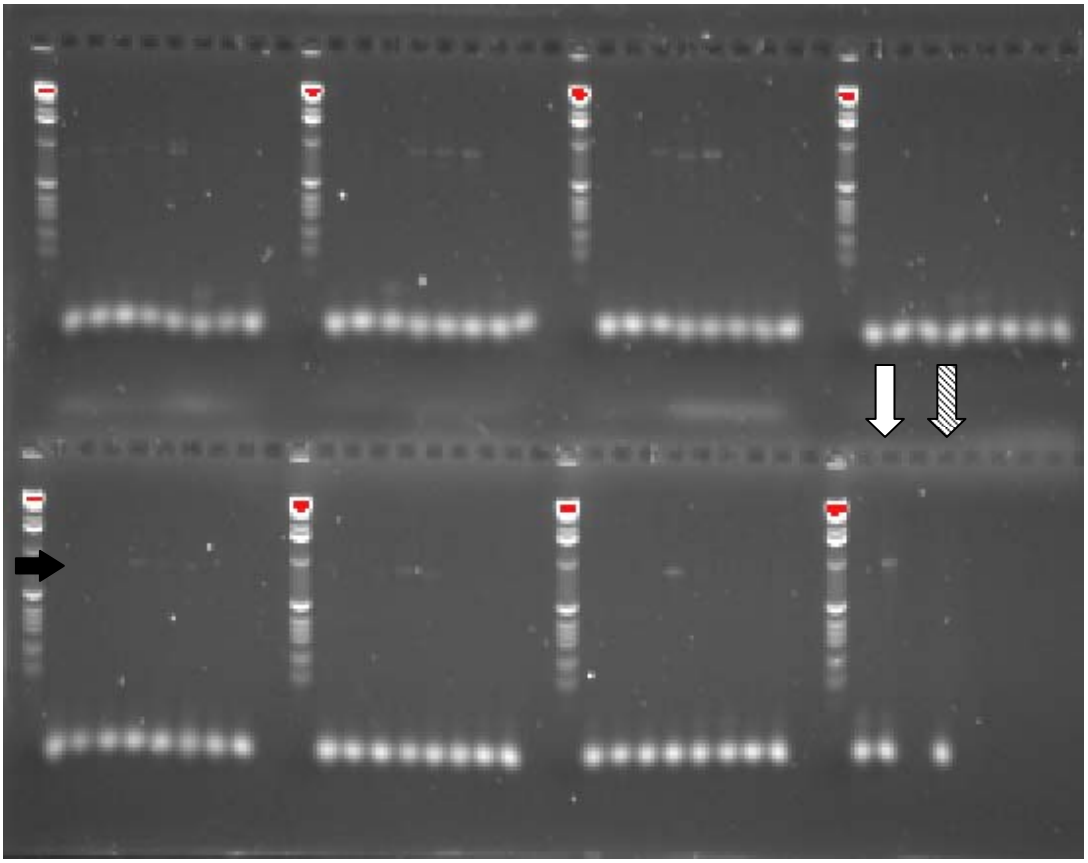

Figure 3.13 A 1 % agarose gel of PCR products amplified from Wollemi Pine roots using the ITS1/ITS4 primer pair. Of the 56 samples, 24 produced bands of approximately 600 bp (black arrow). Note the positive control lane (white arrow) and the negative control lane (arrow with diagonal hatch lines).

Table 3.3 NCBI database matches to nucleotide sequences of fungal DNA amplified from Wollemi Pine roots. Note that plants 5, 14, 40, and 44 were planted with no additional inoculum, plants 10, 21, 23, and 36 were planted with colonized leek roots, and plants 51 and 53 were planted with commercial potting mix containing fungal propagules.

Plant #	Database taxon match	Sequence similarity (%)	Query Coverage (%)	Accession number(s) of best match in database
5	*Cylindrocarpon pauciseptatum*	99	81	EU983277.1 EF607080.1-EF607090.1
	Cylindrocarpon sp.	99	92	AB369414.1 AB369260.1
10	*Cylindrocarpon pauciseptatum*	99	88	EU983277.1 EF607080.1-EF607090.1
	Cylindrocarpon sp.	99	92	AB369414.1 AB369260.1
14	*Cylindrocarpon pauciseptatum*	99	82	EU983277.1 EF607080.1-EF607090.1
	Cylindrocarpon sp.	99	87	AB369414.1 AB369260.1
21	*Phialocephala fortinii*	98	99	AY394921.1 EU103612.1 AY078143.1 EF446148.1
23	*Phialocephala fortinii*	98	100	AY394921.1 EU103612.1 EF093161.1
36	*Cylindrocarpon* sp.	99	93	AB369414.1 AB369260.1
	Cylindrocarpon pauciseptatum	98	88	EU983277.1 EF607080.1-EF607090.1
40	*Cylindrocarpon* sp.	98	92	DQ682573.1 AB369414.1 AB369260.1
	Cylindrocarpon pauciseptatum	99	81	EU983277.1 EF607080.1-EF607090.1
44	*Phialocephala fortinii*	98	100	AY394921.1 EU103612.1 EF093161.1
51	*Phialocephala fortinii*	98	99	AY394921.1 AY078143.1 EU103612.1
53	*Cylindrocarpon pauciseptatum*	98	79	EU983277.1 EF607080.1-EF607090.1

3.3.3 Arbuscular Mycorrhizal Fungi of Native Grasses That Were a Potential Inoculum Source for Wollemi Pine

All of the bluebunch wheatgrass root samples from the study area exhibited heavy arbuscular mycorrhizal colonization (86 % to 100 %). The roots contained distinct arbuscules, vesicles, and hyphae typical of arbuscular mycorrhizas (Figure 3.14). PCR reactions with the ITS1/ITS4 primer pair provided very few weakly amplified products from the grass roots, while the NS31/AM1 primer pair resulted in amplification of a 550 bp product for 16 of the 24 grass samples (Figure 3.15). Eight of the amplified products produced good quality sequences that were BLAST-searched using the NCBI database. Seven of these sequences were similar to *G. intraradices*, but the bootstrap support for this grouping was poor (Figure 3.16). However, strong bootstrap support indicates that one of the grass sample sequences was similar to that of *G. fasciculatum*.

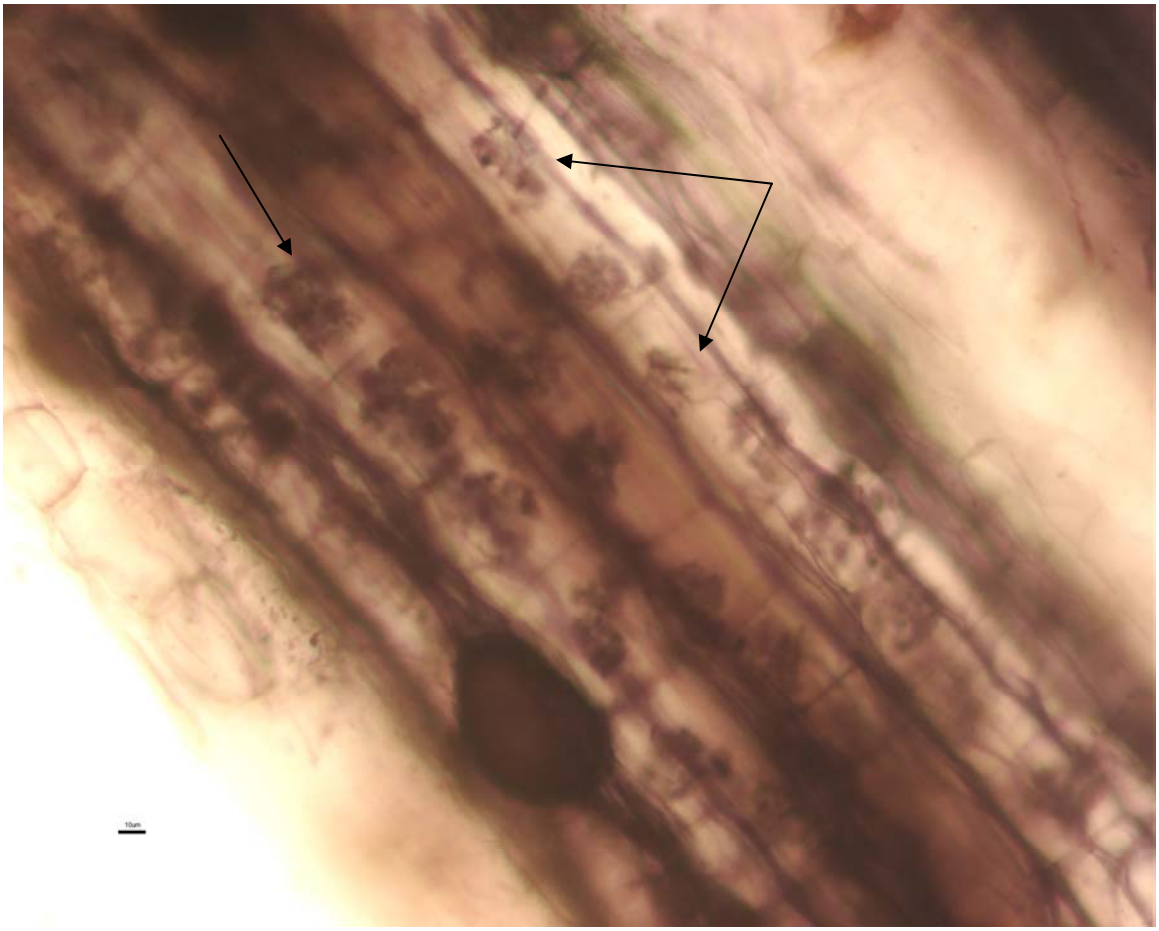

Figure 3.14 A bluebunch wheatgrass root stained with chlorazol black E and viewed at 20X magnification (Bar = 10 μm). Note the intracellular arbuscules formed by the fungal symbiont (indicated by the arrows).

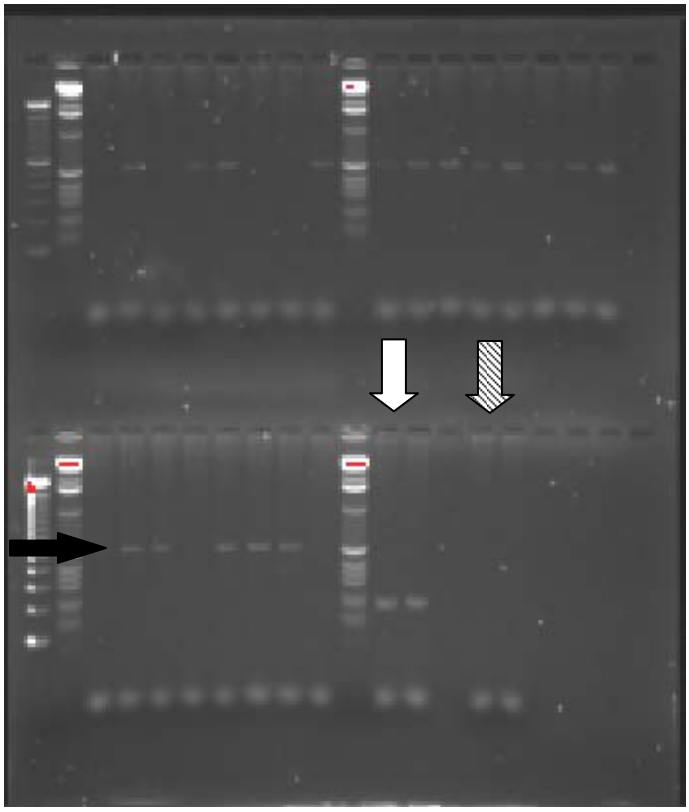

Figure 3.15 A 1 % agarose gel of PCR products amplified from bluebunch wheatgrass root DNA using the NS31/AM1 primer pair. Of the 24 samples, 16 produced clear bands approximately 550 bp in length (black arrow). Note the two positive control lanes (white arrow) and the two negative control lanes (arrow with diagonal hatch lines).

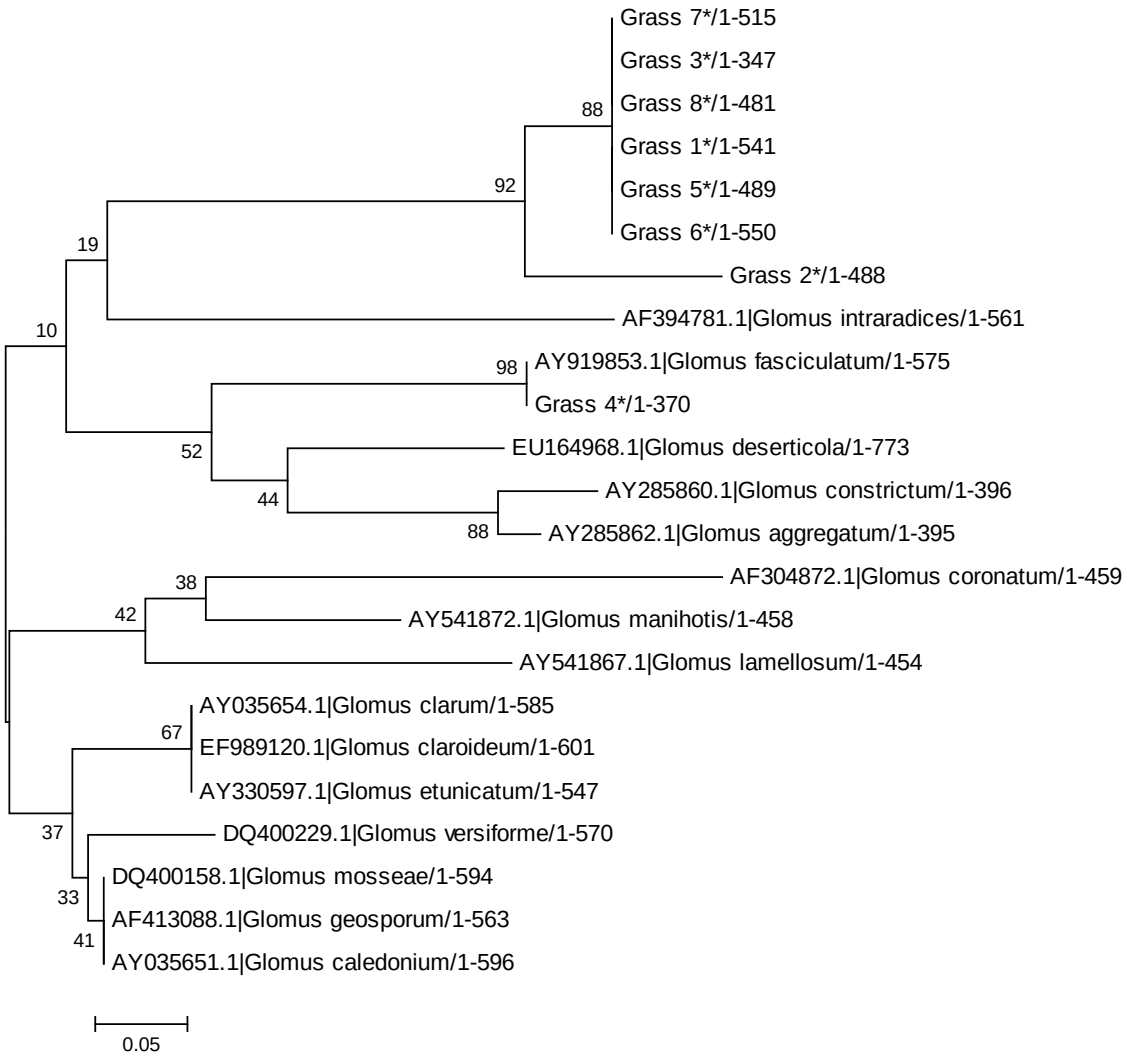

Figure 3.16 Phylogenetic tree depicting sequence similarity of *Glomus* spp. in bluebunch wheatgrass root samples (marked with an asterisk) with known *Glomus* voucher specimens.

3.3.4 Growth of Outplanted Wollemi Pines

All 56 plants survived the length of the experiment and no effects of the fungi or the planting environment were observed. The total increases in plant height over the four month study period were not significantly different between the three treatments ($P = 0.52$, F =

0.65, df = 55) (Table 3.4). Also, the extension rates did not significantly differ between treatments (P = 0.29, F = 1.27, df = 55) (Table 3.4).

Table 3.4 Mean total growth increment and growth rates by treatment of inoculated Wollemi Pines over the four month study period. Note that all mean treatment growth increments and growth rates did not significantly differ by one-way ANOVA at $\alpha = 0.05$. P values for the one-way ANOVAs are given.

Inoculum Treatment	Shoot increment over 4 months (cm) (mean +/- std error)	Growth rate (cm/month) (mean +/- std error)
No additional inoculum	4.80 +/- 0.22	1.07 +/- 0.04
Leek roots colonized by *Glomus intraradices*	5.20 +/- 0.19	1.21 +/- 0.04
Commercial *G. intraradices* inoculum	4.90 +/- 0.79	1.17 +/- 0.20
	P = 0.52	P = 0.29

3.3.5 Nutrient Composition of Outplanted Wollemi Pines

Evaluations of nutrient and mineral concentrations of Wollemi Pines exposed to three different forms of arbuscular mycorrhizal inoculum revealed several interesting trends. Plants not supplied with additional inoculum had significantly increased iron concentration in shoots as compared to plants inoculated with colonized leek roots (P = 0.044, F = 3.56, df = 23) (Figure 3.17). Significantly more manganese ((P = 0.038, F = 3.75, df = 23) (Figure

3.18), calcium (P = 0.011, F = 5.13, df = 23) (Figure 3.19), and zinc (P = 0.045, F = 3.53, df = 23) (Figure 3.20) accumulated in shoot tissue of Wollemi Pines inoculated with commercial inoculum than those inoculated with colonized leek roots. Shoot tissue of plants exposed to commercial inoculum had significantly lower phosphorus (P = 0.024, F = 4.37, df = 23) (Figure 3.21), protein (P = 0.026, F = 4.27, df = 23) (Figure 3.22), and nitrogen (P = 0.021, F = 4.56, df = 23) (Figure 3.23) concentrations as compared to the other two treatments.

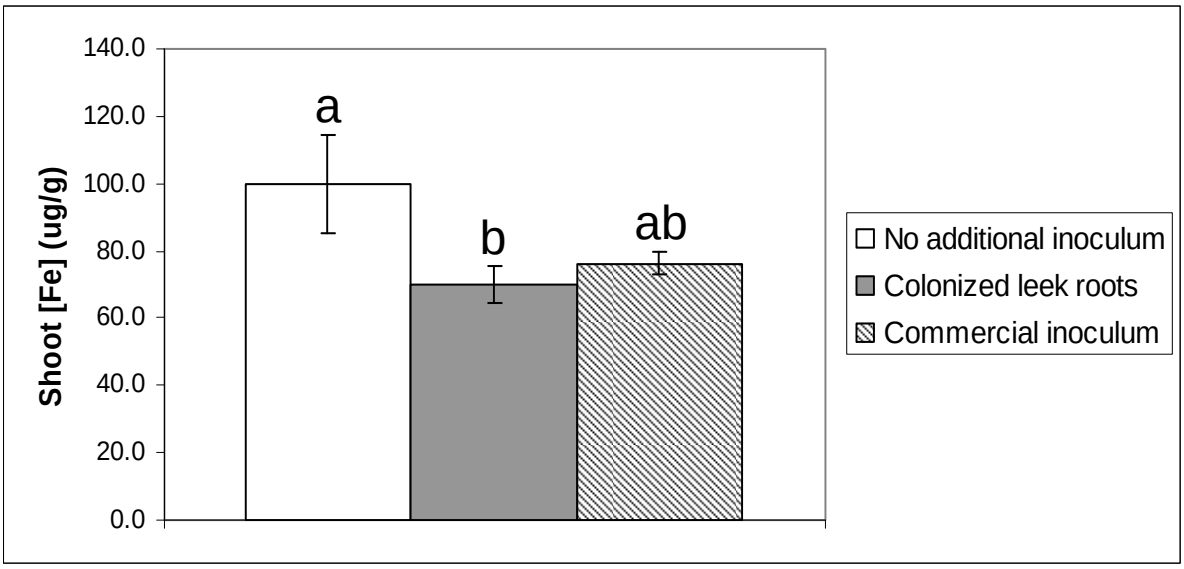

Figure 3.17 Shoot iron concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at α = 0.05 using Duncan's Multiple Range test.

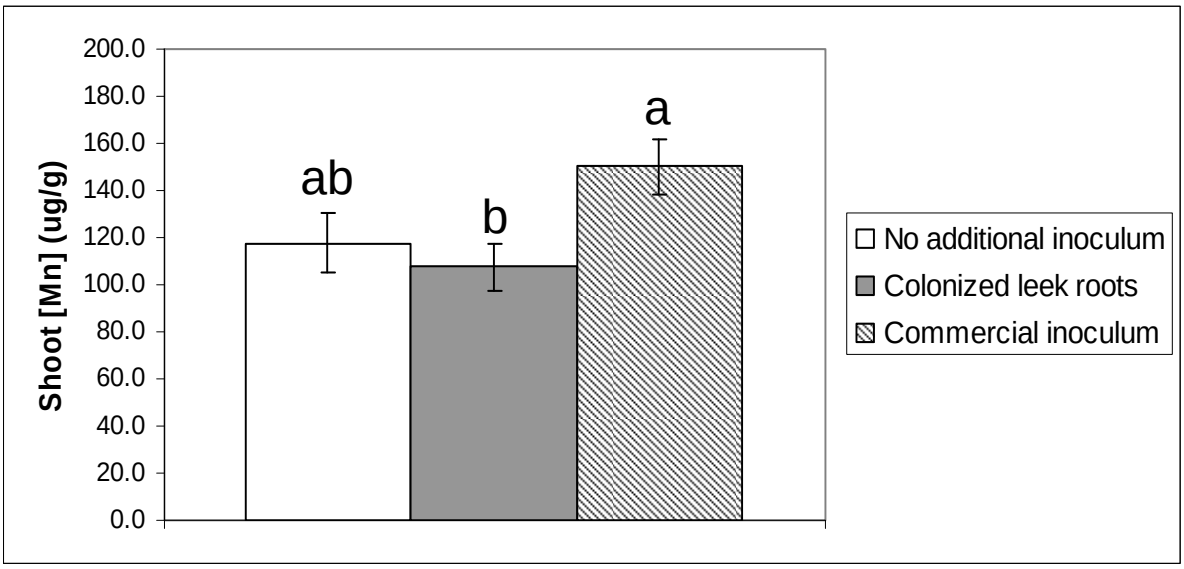

Figure 3.18 Shoot manganese concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

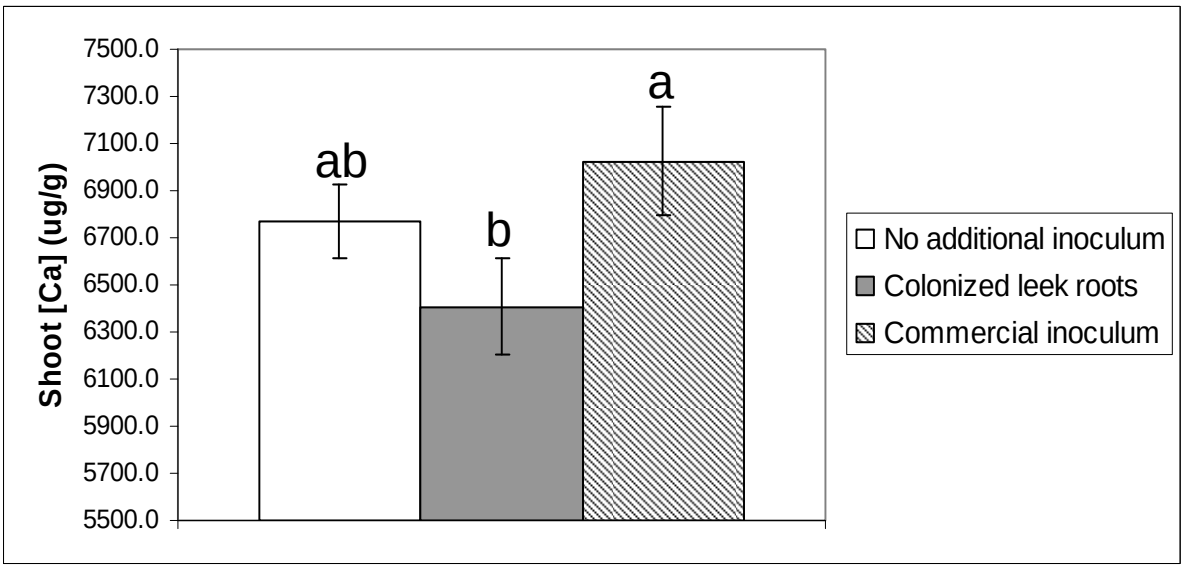

Figure 3.19 Shoot calcium concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

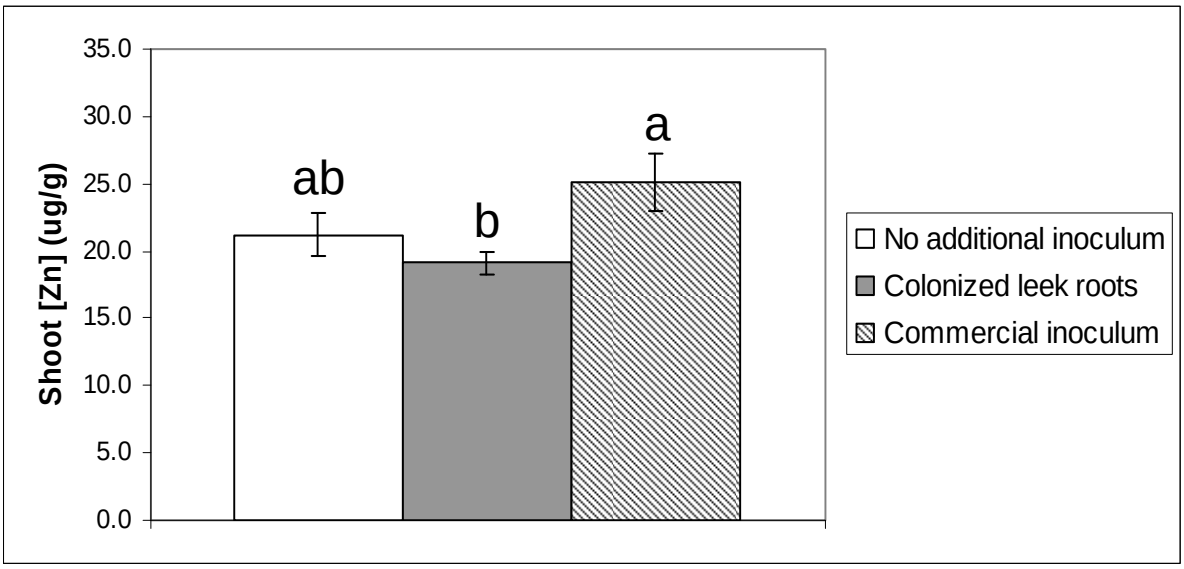

Figure 3.20 Shoot zinc concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

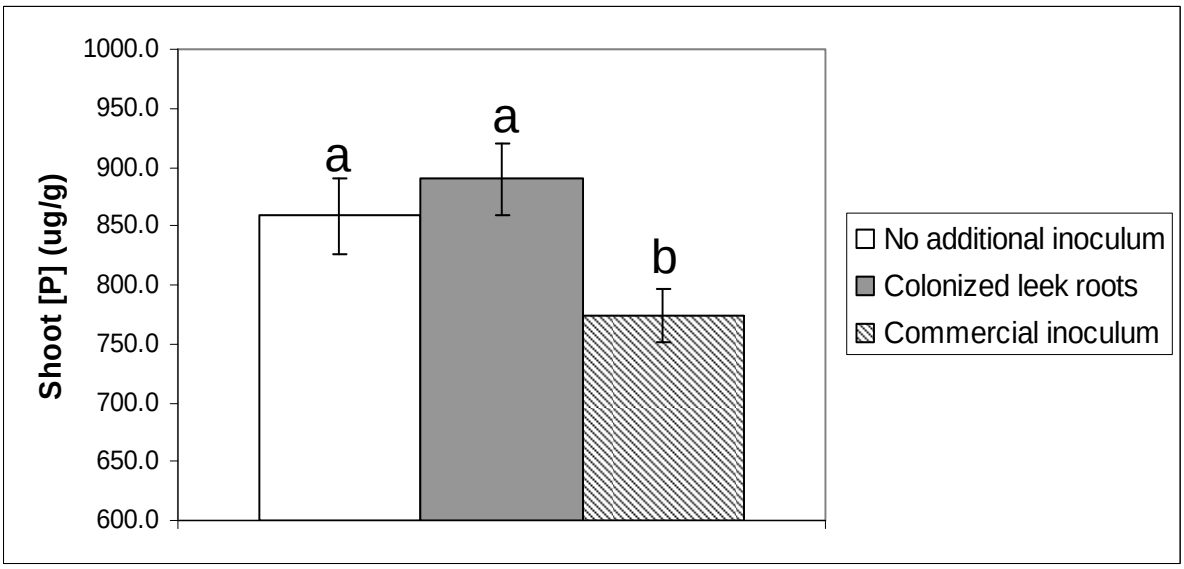

Figure 3.21 Shoot phosphorus concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

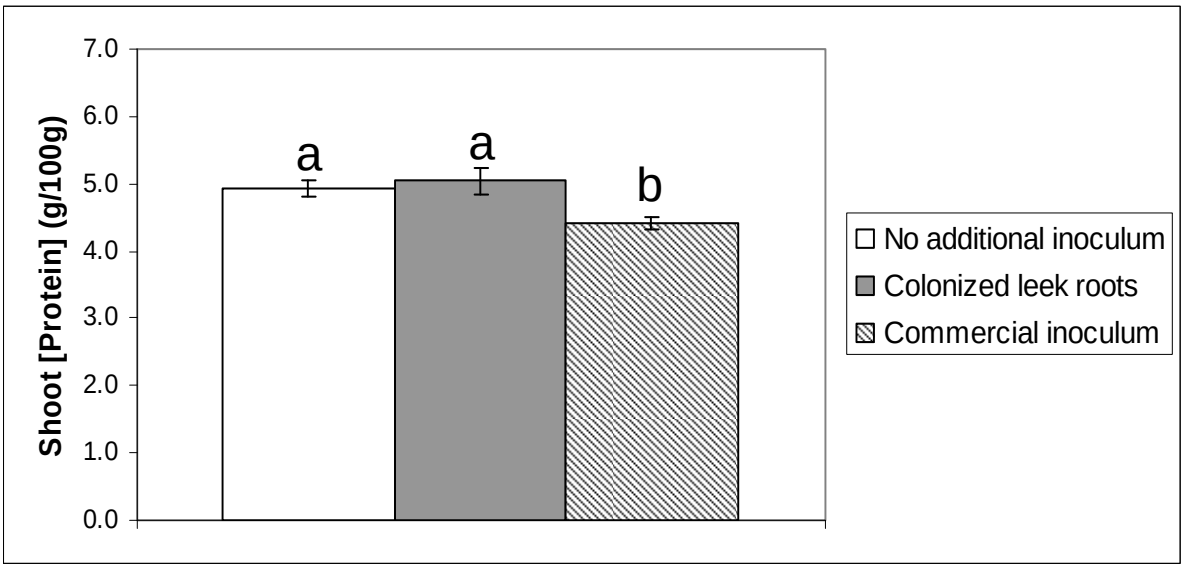

Figure 3.22 Shoot protein concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

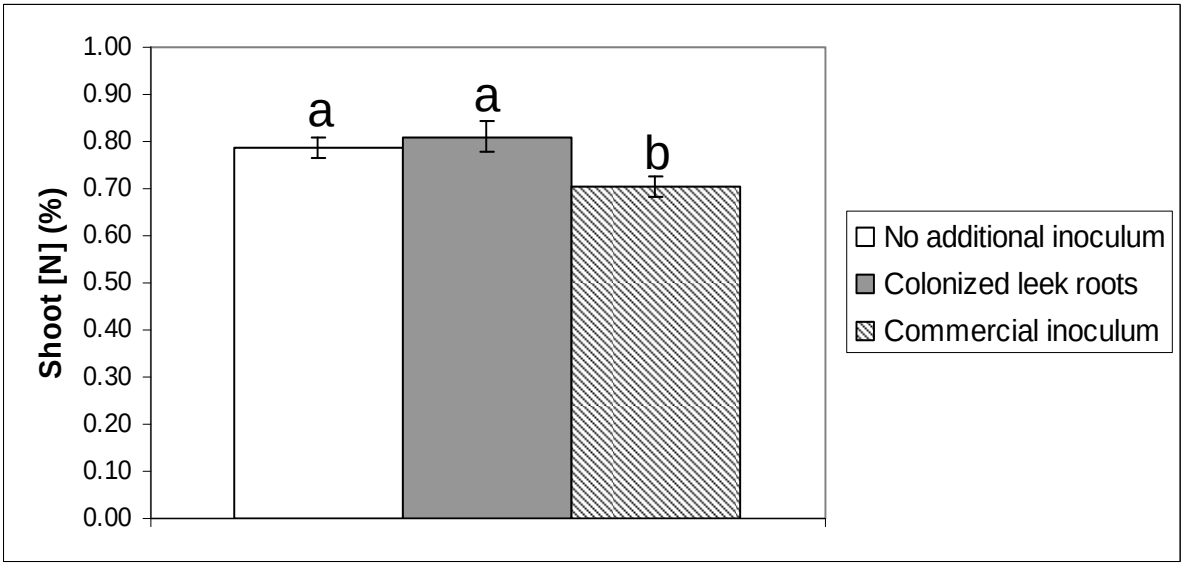

Figure 3.23 Shoot nitrogen concentration of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

Important trends were also revealed when the data was expressed as total ion or nutrient content per plant. Inoculating with colonized leek roots significantly reduced the total shoot iron content as compared to plants that were not supplied any additional inoculum ($P = 0.043$, F = 3.75, df = 23) (Figure 3.24). Addition of a commercial product containing *G. intraradices* propagules significantly increased the total shoot manganese content ($P = 0.046$, F = 3.27, df = 23) (Figure 3.25) and the total shoot zinc content ($P = 0.039$, F = 3.68, df = 23) (Figure 3.26) as compared to plants that were inoculated with colonized leek roots.

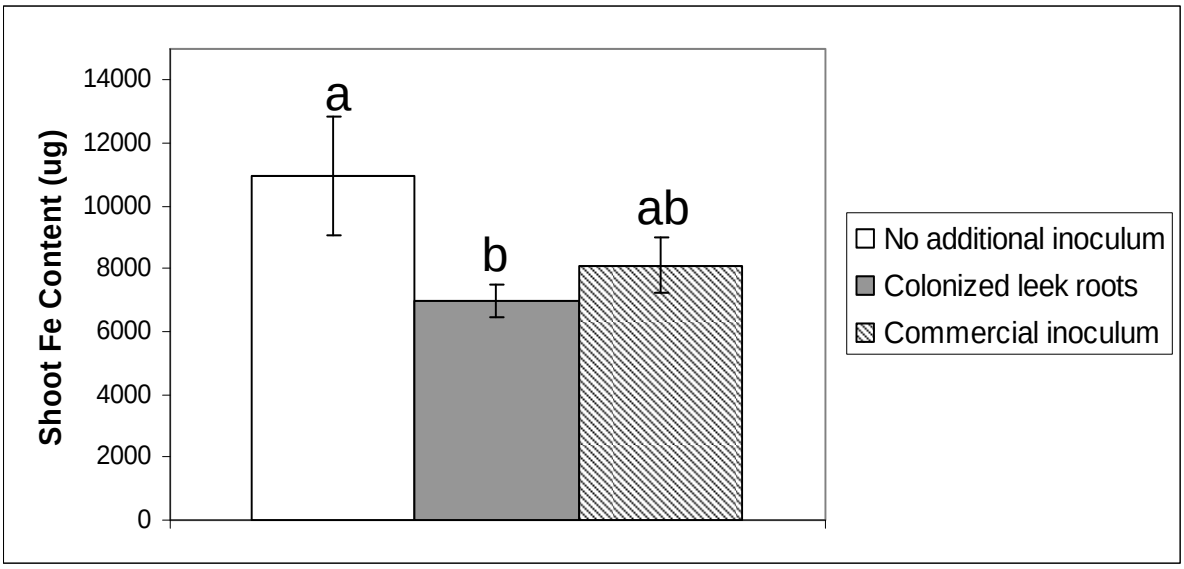

Figure 3.24 Total iron content in shoot tissue of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

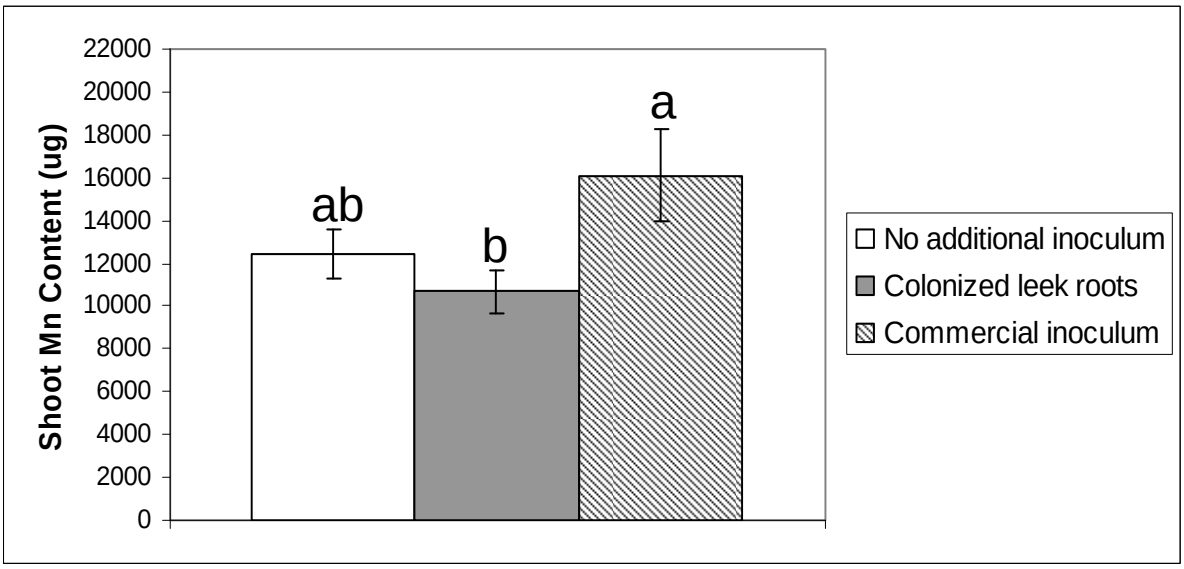

Figure 3.25 Total manganese content in shoot tissue of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

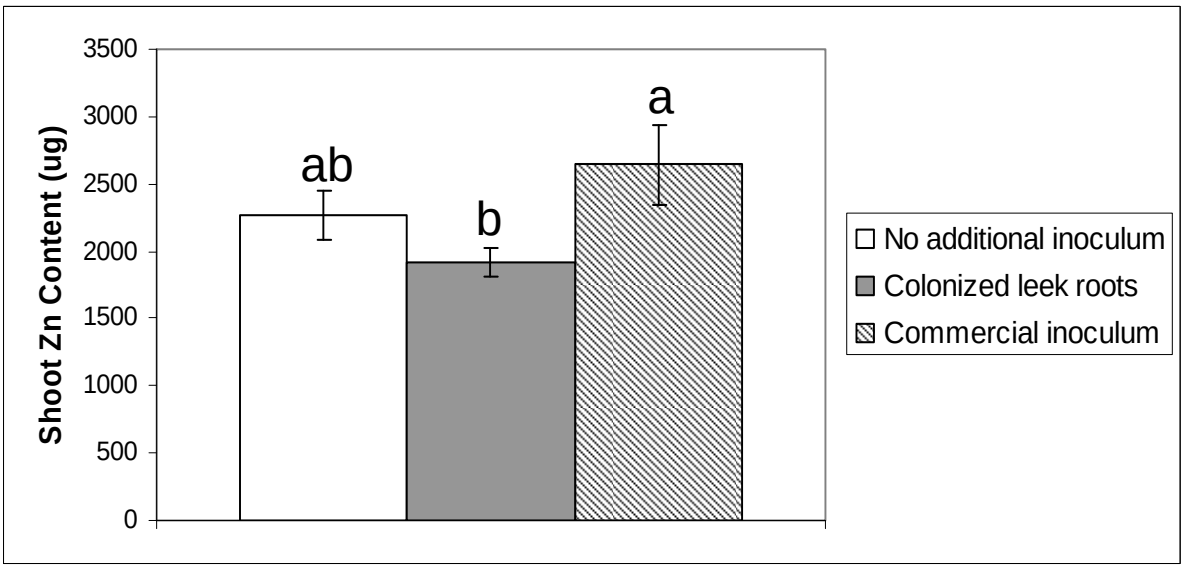

Figure 3.26 Total zinc content in shoot tissue of *Wollemia nobilis* plants inoculated with local and supplemented mycorrhizal fungi. Bars with different letters are significantly different at $\alpha = 0.05$ using Duncan's Multiple Range test.

3.4 Discussion

To date, McGee et al. (1999) have published the only research study investigating Wollemi Pine mycorrhizal associations in a natural forest environment. Their work involved sampling Wollemi Pine roots on two separate occasions from two mature trees in Wollemi National Park (McGee et al., 1999). Microscopic examination of the roots revealed colonization by arbuscular mycorrhizal fungi, as well as one instance of ectendomycorrhizal colonization on one root (McGee et al., 1999). While ectendomycorrhizas are relatively rare associations (Yu et al., 2001), arbuscular mycorrhizas are widespread and have been commonly detected in the Araucariaceae (e.g. Morrison and English, 1967; Zandavalli et al., 2004). The work of McGee et al. (1999) was the impetus for the current research study. In the current study, Wollemi Pines were planted in a ponderosa pine forest and inoculated with local or commericially-supplied arbuscular mycorrhizal fungi. The effects of the inoculation treatments on *W. nobilis* colonization, growth, and nutrition were compared.

The most significant finding of this study was that mycorrhizal colonization was not observed on the Wollemi Pine roots despite abundant sources of inoculum (e.g. *Glomus* spp. in native grasses, colonized leek roots, and commercial inoculum) that were in close proximity to the plants. A possible reason for the lack of colonization may be due to the relatively short time span of this study. Arbuscular mycorrhizal colonization typically occurs in eight to ten weeks in laboratory experiments, but field studies do not necessarily adhere to this time frame (Ferguson and Woodhead, 1982; Ning and Cumming, 2001). In addition, the expected time for colonization to occur varies among taxa and has not been evaluated for Wollemi Pines. McGee et al. (1999) only detected colonization in roots from mature

established Wollemi Pines, so it is possible that the four month study period was not long enough for the roots to become colonized or that colonization occurs in slightly older trees.

Some aspects of the study area may have affected the ability of the roots to become colonized. Wollemi Pines naturally grow in a temperate rainforest gorge sheltered by cliffs and tall trees (Jones et al., 1995), which is quite different from the exposed planting area experienced in the current study. The recorded climate and soil moisture data exemplifies the hot and dry conditions that the Wollemi Pines were exposed to throughout the study. Annual temperatures in Wollemi National Park range from 4 to 31 ^{o}C, but lack of sunlight may further reduce these temperatures for the natural Wollemi Pine population (NPWS, 1998). Also, average rainfall in Wollemi National Park is 40 to 80 mm per month (NPWS, 1998), which is much higher than was experienced in the current study. Although the soil that supports Wollemi Pine growth in Australia has poor structure and low water retention (Beadle, 1962; NPWS, 1998), the trees grow adjacent to a creek that flows all year and provides the tree roots with some moisture (Jones et al., 1995). In addition, the PAR measurements taken at each plant are indicative of the intense sun exposure and minimal shading many plants experienced during the study, which is in contrast to their natural surroundings. Only 10 % of ambient light reaches the native Wollemi Pine population, and this amount may be further reduced by shading from competing trees (NPWS, 1998).

The mycorrhizal fungal species present in the current study may have been unsuitable for colonizing Wollemi Pines. Although arbuscular mycorrhizal associations are generally unspecific (Peterson et al., 2004), Wollemi Pine symbiont specificity has not been determined and compatible mycobionts have not been identified. The bluebunch wheatgrass roots at the study area were heavily colonized by *Glomus* spp. closely related to *G.*

intraradices and *G. fasciculatum*, but it is not known if these fungi form associations with Wollemi Pines or other members of the Araucariaceae. However, several different *Glomus* spp. have been shown to colonize roots of some plants in the Araucariaceae, including *G. clarum*, *G. macrocarpum*, *G. etunicatum*, and *G. geosporum* in Brazilian Pine (*Araucaria angustifolia*) (Breuninger et al., 2000; Moreira-Souza et al., 2003; Zandavalli et al., 2004).

There are few studies profiling the mycorrhizal fungi colonizing grass roots, particularly bluebunch wheatgrass roots. However, there is evidence of multiple *Glomus* species colonizing a variety of grasses, including *Festuca pratensis* (Santos et al., 2006), *Lolium perenne* (Gollotte et al., 2004), and *Ammophila arenaria* (Kowalchuk et al., 2002), but these fungi are often not identified beyond the genus level. In the current study, more intensive grass root sampling for molecular identification purposes may have resulted in a broader range of sequences and perhaps more accurate taxonomic matches with specific fungi. In addition, further attempts to identify mycorrhizal fungi colonizing grass roots may incorporate a cloning step prior to sequencing in order to separate sequences from multiple species that are present in the samples (e.g. Redecker, 2000; Vandenkoornhuyse et al., 2002). Cloning PCR products originating from field samples would permit isolation and multiplication of the desired sequence from each organism, thus producing multiple separate sequences of improved quality. Therefore, further research is required first to identify the *Glomus* species colonizing the grass roots, as they were not compatible symbionts with Wollemi Pines in the current study, and then to determine fungi that do form associations with *W. nobilis* roots.

Interestingly, endophytic fungal structures were detected in every Wollemi Pine root system. Leaf and stem endophytic fungi of Wollemi Pines have been described in the

context of taxol production (Strobel et al., 1997), but there are no published accounts of root endophytes in *W. nobilis*. Two endophyte taxa proved to be strong taxonomic matches to the fungal DNA sequences isolated from Wollemi Pine roots; however, had more root tissue been analyzed, the number of taxa detected in the samples may also have increased. The most common endophyte detected in Wollemi Pine roots from each treatment was *Cylindrocarpon* sp., which is a ubiquitous soil-borne fungus associated with the rhizosphere of many plant species (Traquair, 1995). The taxon that commonly matched with DNA from Wollemi Pine roots was *C. pauciseptatum*, which is a recently described species that is closely related to the root rot-causing *C. destructans* (Schroers et al., 2008). *Cylindrocarpon* spp. are commonly saprobes in the soil or weak pathogens infecting roots and stems of various plant hosts (Schroers et al., 2008). However, the role of these fungi in plant health is not fully determined, as they have been isolated from both healthy and diseased plants (Dahm and Strzelczyk, 1987).

The other fungal taxon frequently identified in Wollemi Pine roots of all treatments was *Phialocephala fortinii*, which is a dark septate fungal endophyte common in Pinaceae roots but is also found in many herbaceous shrubs and trees (Grünig et al., 2002; Grünig et al., 2008a; Grünig et al., 2008b). This fungus colonizes roots with melanized hyphae, and forms microsclerotia and a weak Hartig net in some conifers (Horton et al., 1998). The microsclerotia develop inter- and intracellularly within the cortex of the root, and are composed of compact, often irregularly lobed hyphae (O'Dell et al., 1993). The functional role of *P. fortinii* has not been determined, as there is evidence of beneficial, neutral, or pathogenic interactions with the host depending on the growing conditions, fungal strains, and plant species (Grünig et al., 2008a). Although there was no evidence of pathogenic

effects of these fungi on the Wollemi Pines, further research is required to determine the nature of the association with these root endophytic fungi.

Despite a lack of observed mycorrhizal colonization in Wollemi Pine roots, shoot concentrations of several nutritional parameters differed significantly between fungal treatments. The soil samples taken from the study area indicate that these differences were likely not due to inconsistencies in nutrient content of the soil. Therefore, the observed treatment differences in nutrient and mineral parameters may have been caused by the inoculum that the trees were exposed to. It is important to point out that the trees planted with commercial growth medium were likely provided with improved nutrient sources in close proximity to the roots, which may have caused some of the observed differences. Also, arbuscular mycorrhizal fungi are typically characterized as obligate biotrophs that require root colonization to survive (Williams, 1992), but there have been studies demonstrating that arbuscular mycorrhizal inoculum can affect plant roots without colonization taking place. For example, improved rooting and increased nutrient uptake of miniature rose cuttings were observed following inoculation with *Glomus intraradices* but prior to colonization (Scagel, 2001; Scagel, 2004). These observed plant responses may be associated with changes in the secondary metabolism of the plants following inoculation (Larose et al., 2002), but this notion has not been fully explored. Without autoclaved inoculum included as a control treatment in this study (Koide and Li, 1989), there is not enough information to definitively state whether the inoculum affected the nutrient composition of the trees. Further research is required to explain the effects of mycorrhizal inoculum on Wollemi Pine roots in the absence of colonization.

3.5 Conclusion

This is the first study of its kind inoculating Wollemi Pines with mycorrhizal fungi in a forest environment. The results demonstrate the difficulty in studying a plant outside of its natural range, particularly a recently discovered rare plant that is not well-understood. The Wollemi Pine is unique from other conifers, even from those in the Araucariaceae, which creates difficulty in predicting and explaining experimental outcomes. The lack of mycorrhizas detected in Wollemi Pines growing in an inoculum-rich environment presents many questions, particularly regarding the suitability of the environment and fungal symbionts. At the same time, this study provides an interesting account of a rainforest species surviving and growing in desert-like conditions. More work is required to evaluate the effects of Wollemi Pine root endophytes on plant health and nutrient relations. The potential effects of the mycorrhizal inoculum on *W. nobilis* tissue nutrient concentrations in the absence of colonization also require further exploration. Future studies must look at the interactions between mycorrhizal fungi and Wollemi Pines in a field setting to determine the relevance of incorporating the symbiosis into conservation research.

3.6 References

Altschul SF; Madden TL; Schaffer AA; Zhang J; Zhang Z; Miller W; Lipman DJ. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25: 3389-3402

Bardell D. (2006) The discovery of a tree (*Wollemia nobilis*) from the age of the dinosaurs. Bios. 77: 20-23

Beadle NCW. (1962) Soil phosphate and the delimitation of plant communities in eastern Australia II. Ecology. 43: 281-288

Breuninger M; Einig W; Magel E; Cardoso E; Hampp R. (2000) Mycorrhiza of Brazil Pine (*Araucaria angustifolia* [Bert. O. Ktze.]). Plant Biology. 2: 4-10

Brundrett MC; Piché Y; Peterson RL. (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can. J. Bot. 62: 2128-2134

Bullock S; Summerell BA; Gunn LV. (2000) Pathogens of the Wollemi Pine, *Wollemia nobilis*. Australasian Plant Pathology. 29: 211-214

Carter MR. (1993) Soil sampling and methods of analysis. Boca Raton, Florida: CRC Press Inc

Dahm H; Strzelczyk E. (1987) Cellulolytic and pectolytic activity of *Cylindrocarpon destructans* (Zins.) Scholt. isolates pathogenic and non-pathogenic to Fir (*Abies alba* Mill.) and Pine (*Pinus sylvestris* L.). Journal of Phytopathology. 118: 76-83

Ferguson JJ; Woodhead SH. (1982) Production of endomycorrhizal inoculum. A. Increase and maintenance of vesicular-arbuscular mycorrhizal fungi. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 47-53

Gardes M; Bruns TD. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology. 2: 113-118

Gollotte A; van Tuinen D; Atkinson D. (2004) Diversity of arbuscular mycorrhizal fungi colonizing roots of the grass species *Agrostis capillaries* and *Lolium perenne* in a field experiment. Mycorrhiza. 14: 111-117

Grünig CR; Sieber TN; Rogers SO; Holdenrieder O. (2002) Spatial distribution of dark septate endophytes in a confined forest plot. Mycological Research. 106: 832-840

Grünig CR; Duò A; Sieber TN; Holdenrieder O. (2008a) Assignment of species rank to six reproductively isolated cryptic species of the *Phialocephala fortinii* s.l.-*Acephala applanta* species complex. Mycologia. 100: 47-67

Grünig CR; Queloz V; Sieber TN; Holdenrieder O. (2008b) Dark septate endophytes (DSE) of the *Phialocephala fortinii* s.l. – *Acephala applanta* species complex in tree roots: classification, population biology, and ecology. Botany. 86: 1355-1369

Helgason T; Daniell TJ; Husband R; Fitter AH; Young JPW. (1998) Ploughing up the wood-wide web? Nature. 394: 431

Horton TR; Cázares E; Bruns TD. (1998) Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. Mycorrhiza. 8: 11-18

Horwitz W. (2000) Official methods of analysis of AOAC international, 17th Ed. Gaithersburg, Maryland: Association of Official Analytical Chemists

Jones WG; Hill KD; Allen JM. (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. Telopea. 6: 173-176

Koide RT; Li M. (1989) Appropriate controls for vesicular-arbuscular mycorrhiza research. New Phytologist. 111: 35-44

Kovács GM; Rudnóy S; Vágvölgyi C; Lásztity D; Rácz I; Bratek Z. (2001) Intraspecific invariability of the internal transcribed spacer region of rDNA of the truffle *Terfezia terfezioides* in Europe. Folia Microbiologica. 46: 423-426

Kowalchuk GA; de Souza FA; van Veen JA. (2002) Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. Molecular Ecology. 11: 571-581

Larose G; Chênevert R; Moutoglis P; Gagné S; Piché Y, Vierheilig H. (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. Journal of Plant Physiology. 159: 1329-1339

Lloyd D; Angrove K; Hope G; Thompson C. (1990) A guide to site identification and interpretation for the Kamloops forest region. Victoria, B.C., Canada: Research Branch, B.C. Ministry of Forests

Martin KJ; Rygiewicz PT. (2005) Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology. 5: 28-38

McGee PA; Bullock S; Summerell BA. (1999) Structure of mycorrhizae of the Wollemi pine (*Wollemia nobilis*) and related Araucariaceae. Aust. J. Bot. 47: 85-95

McGonigle TP; Miller MH; Evans DG; Fairchild GL; Swan JA. (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist. 115: 495-501

Menge JA, Timmer LW. (1982) Procedure for inoculation of plants with vesicular-arbuscular mycorrhizae in the laboratory, greenhouse, and field. In: Methods and principles of mycorrhizal research. Schenck NC. The American Phytopathological Society. 59-68

Moreira-Souza M; Trufem SFB; Gomes-da-Costa SM; Cardoso EJBN. (2003) Arbuscular mycorrhizal fungi associated with *Araucaria angustifolia* (Bert.) O. Ktze. Mycorrhiza. 13: 211-215

Morrison TM; English DA. (1967) The significance of mycorrhizal nodules of *Agathis australis*. New Phytologist. 66: 245-250

Ning J; Cumming JR. (2001) Arbuscular mycorrhizal fungi alter phosphorus relations of broomsedge (*Andropogon virginicus* L.) plants. Journal of Experimental Biology. 52: 1883-1891

NPWS (National Parks and Wildlife Service). (1998) Wollemi Pine Recovery Plan. Sydney, New South Wales: NPWS

O'Dell TE; Massicotte HB; Trappe JM. (1993) Root colonization of *Lupinus latifolius* Agardh. and *Pinus contorta* Dougl. by *Phialocephala fortinii* Wang & Wilcox. New Phytologist. 124: 93-100

Offord C; Meagher P. (2006) Wollemi Pine: from the wild to the world. Chronica Horticulturae. 46: 10-13

Peakall R; Ebert D; Scott LJ; Meagher PF; Offord CA. (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). Molecular Ecology. 12: 2331-2343

Peterson RL; Massicotte HB; Melville LH. (2004) Mycorrhizas: anatomy and cell biology. Ottawa, Ontario: NRC Research Press.

Redecker D. (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza. 10: 73-80

Santos JC; Finlay RD; Tehler A. (2006) Molecular analysis of arbuscular mycorrhizal fungi colonizing a semi-natural grassland along a fertilization gradient. New Phytologist. 172: 159-168

Scagel CF. (2001) Cultivar specific effects of mycorrhizal fungi on the rooting of miniature rose cuttings. Journal of Environmental Horticulture. 19: 15-20

Scagel CF. (2004) Changes in cutting composition during early stages of adventitious rooting of miniature rose altered by inoculation with arbuscular mycorrhizal fungi. Journal of the American Society for Horticultural Science. 129: 624-634

Schroers H-J; Žerjav M; Munda A; Halleen F; Crous PW. (2008) *Cylindrocarpon pauciseptatum* sp. nov., with notes on *Cylindrocarpon* species with wide, predominantly 3-septate macroconidia. Mycological Research. 112: 82-92

Schüβler A; Schwarzott D; Walker C. (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycological Research. 105: 1413-1421

Simon L; Lalonde M; Bruns TD. (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Applied and Environmental Microbiology. 58: 291-295

Smith SE; Read DJ. (1997) Mycorrhizal symbiosis. 2nd Ed. San Diego, California: Academic Press, Inc.

Strobel GA; Hess WM; Li J; Ford E; Sears J; Sidhu RS; Summerell B. (1997) *Pestalotiopsis guepinii*, a taxol-producing endophyte of the Wollemi pine, *Wollemia nobilis*. Aust. J. Bot. 45: 1073-1082

Traquair JA. (1995) Fungal biocontrol of root diseases: endomycorrhizal suppression of cylindrocarpon root rot. Can. J. Bot. 73 (Suppl. 1): S89-S95

Vandenkoornhuyse P; Husband R; Daniell TJ; Watson IJ; Duck JM; Fitter AH; Young JPW. (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Molecular Ecology. 11: 1555-1564

Vandenkoornhuyse P; Ridgway KP; Watson IJ; Fitter AH; Young JPW. (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. Molecular Ecology. 12: 3085-3095

Williams PG. (1992) Axenic culture of arbuscular mycorrhizal fungi. Methods in Microbiology. 24: 203-220

Yu TEJ-C; Egger KN; Peterson RL. (2001) Ectendomycorrhizal associations – characteristics and functions. Mycorrhiza. 11: 167-177

Zandavalli RB; Dillenburg LR; de Souza PVD. (2004) Growth response of *Araucaria angustifolia* (Araucariaceae) to inoculation with the mycorrhizal fungus *Glomus clarum*. Applied Soil Ecology. 25: 245-255

Chapter Four: Allelopathic Effects of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) Soil Extract on Leek and Sorghum Seed Germination[3]

4.1 Introduction

Allelopathy refers to the chemical inhibition of one species by another. The inhibitory chemical is released into the environment where it affects the development and growth of neighbouring plants. A diverse array of phytochemicals is responsible for these interactions, including structures that range from simple hydrocarbons to complex aromatics (Putnam, 1988; Czarnota et al., 2003). Phytotoxic effects of these chemicals include disruption of DNA replication, interference with phytohormone activity, and obstruction of electron transport in chloroplasts and mitochondria (e.g. Rasmussen et al., 1992; Einhellig et al., 1993). Although allelopathic interactions have been detected in a variety of ecosystems (e.g. Mahall and Callaway, 1992), studies have concentrated on the commercial use of allelopathic crop plants in agricultural weed control (Putnam et al., 1983; Weston, 1996).

In addition to crop plants, allelochemical production has been detected in six of seven coniferous plant families (Singh et al., 1999). Allelopathy research in conifers often focuses on coniferous leaf litter and debris biochemically hindering the establishment of other vegetation, particularly in forest regeneration following a disturbance (e.g. Lill and Waid, 1975; Mallik and Newton, 1988). Other studies have focused on the suppression of under-

[3] A version of this chapter will be submitted for publication. Biggs LE; Murch SJ; Durall DM. Allelopathic Effects of Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen) Soil Extract on Leek and Sorghum Seed Germination.

storey plant growth due to allelochemicals secreted by over-storey plants, or vice versa (e.g. Kil and Yim, 1983; Gallet, 1994). The allelopathic potential of an ancient endangered conifer belonging to the Araucariaceae, Wollemi Pine (*Wollemia nobilis* Jones, Hill & Allen), has not been described to date.

The Wollemi Pine was discovered in 1994 in a rainforest gorge of Wollemi National Park near Sydney, Australia (Jones et al., 1995) and is currently restricted to three populations of less than 100 trees in this area (Offord and Meagher, 2006). In a study of the growth of Wollemi Pine with mycorrhizal inoculation via co-culture with infected leeks (*Allium porrum* L.), few of the leek plants survived and none of the leek seeds germinated in the presence of *W. nobilis* (Chapter 2). These observations led to the current study, which was designed to determine if Wollemi Pines exhibit an allelopathic influence on plant species commonly used to culture arbuscular mycorrhizas, such as leek and sorghum (*Sorghum bicolor* (L.) Moench). The specific objective of the study was to quantify and compare leek and sorghum seed germination after exposure to the soil extract from field and laboratory plantings of Wollemi Pine. The hypotheses proposed for this research are: (1) there is a difference in seed germination between sorghum and leek exposed to extracts of soil from Wollemi plants, and (2) differences in pH between the different soil extracts are related to differences in germination of sorghum or leek.

4.2 Materials and Methods

4.2.1 Phytotoxic Effects of Extracts on Sorghum and Leek Seed Germination

To test for allelopathic potential in Wollemi Pine soil extracts, leek (*Allium porrum* L. cv. Giant Musselburgh) and sorghum (*Sorghum bicolor* (L.) Moench) seeds were exposed to extract from three substrates used to grow Wollemi Pines, extracts from soils not used to grow Wollemi Pines, or deionized water (Saito et al., 1993). The experiment was set up as a two by seven factorial design, consisting of the two seed types and seven treatments, with five replicates of each seed/treatment combination. Wollemi Pine soil extract was obtained from the following sources: five replicate field soil samples taken from a Wollemi Pine plantation established in May 2008 in a native ponderosa pine forest in Kelowna, British Columbia; five replicate samples of 2:1 peat/sand from pots that had been used to grow Wollemi Pines at UBC Okanagan for 18 weeks (Sun Gro Horticulture Canada Ltd, Seba Beach, Alberta, Canada); and five replicate samples of Premier PRO-MIX® BX commercial growth medium (Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada) from pots that had been used to grow Wollemi Pines at UBC Okanagan for 30 weeks. Four hundred mL of each soil sample was washed with 200 mL of deionized water and the extract was collected after passing through a cheesecloth filter. Extracts of five replicate samples of each of the above substrates, but that had not been used to grow Wollemi Pines, as well as five replicates of deionized water were used as controls. The pH of the soil and soil extract were measured for each sample using a Beckman 340 pH meter (Beckman Coulter Inc, Fullerton, California, U.S.A.).

Seeds of sorghum and leek were surface-sterilized in 8 % bleach (5.25 % sodium hypochlorite) for 10 minutes and rinsed thoroughly with sterile deionized water (Geil and Guinel, 2002). The seeds were germinated in sterile 10 cm Petri plates. The bottom of each plate was lined with sterile Whatman No. 1 filter paper moistened with 3 mL of the appropriate extract treatment (Panasiuk et al., 1986). Twenty seeds of leek or sorghum were evenly distributed on the filter paper in each plate. The Petri plates were sealed with Parafilm and randomly placed in a Conviron CMP3244 growth chamber at 25 ^{o}C/20 ^{o}C day/night with a 16 hour photoperiod of 250 μmol $m^{-2}s^{-1}$. Germination data was collected at five, ten, and fifteen days after starting the experiment.

4.2.2 Data Analysis

To test for significant differences in germination between the seed types, the Generalized Linear Model (GLM) procedure with Duncan's Multiple Range test (DMRT) was used to perform a two-way analysis of variance (ANOVA) on treatment germination means (SAS software; SAS Institute Inc, Cary, North Carolina, U.S.A.). The main effects were seed type and soil extract type, and the interaction between the two main effects was also evaluated. This procedure was performed using the leek and sorghum germination data collected at the five, ten, and fifteen day stages of the experiment (all n = 70). The same procedure was also used to test for significant differences in pH between Wollemi Pine soil extracts and respective control extracts (n = 10 for each extract type). Linear regression analysis was performed in SAS to determine if differences in soil extract pH values were related to differences in seed germination at each of the data collection periods. This analysis was performed separately for each seed type (each n = 35).

4.3 Results

4.3.1 Germination of Seeds Treated with *Wollemia nobilis* Soil Extract

Leek seed germination was adversely affected by exposure to Wollemi Pine soil extracts as compared to extract from control substrates that were not used to grow Wollemi Pines (Table 4.1; Figure 4.1). Significant differences in seed germination were noted by the fifth day of experimentation (data not shown). Similar results were obtained on days 10 (data not shown) and 15. In contrast to leeks, sorghum seed germination was not affected by treatment with aqueous leachates from Wollemi Pine plantings (Table 4.1; Figure 4.2). After five days, the majority of sorghum seeds had germinated on all experimental plates, and many of the developing plants were beginning to form leaves. As expected, the two-way analysis of variance of mean leek and sorghum germination percentages revealed a significant interaction between seed type and extract treatment at five days ($P<0.0001$, $F = 57.73$, $df = 69$), ten days ($P<0.0001$, $F = 56.63$, $df = 69$), and fifteen days ($P<0.0001$, $F = 55.91$, $df = 69$) following exposure to the treatments.

Table 4.1 Germination of *Allium porrum* and *Sorghum bicolor* seeds fifteen days following treatment with Wollemi Pine soil extracts, control soil extracts, or deionized water. Germination means with different superscripts are significantly different by Duncan's Multiple Range test at $\alpha = 0.05$.

Treatment	Leek Seed Germination (%) (mean ± std error)	Sorghum Seed Germination (%) (mean ± std error)
Field soil (with Wollemi)	19.00 ± 3.32^{b}	90.00 ± 2.24^{a}
Field soil (without Wollemi)	87.00 ± 3.39^{a}	89.00 ± 1.00^{a}
2:1 peat/sand (with Wollemi)	18.00 ± 3.39^{b}	91.00 ± 1.87^{a}
2:1 peat/sand (without Wollemi)	85.00 ± 1.58^{a}	87.00 ± 5.15^{a}
PRO-MIX BX (with Wollemi)	23.00 ± 3.00^{b}	92.00 ± 3.39^{a}
PRO-MIX BX (without Wollemi)	84.00 ± 1.87^{a}	91.00 ± 3.32^{a}
De-ionized water	86.00 ± 4.00^{a}	88.00 ± 3.00^{a}

Figure 4.1 Comparison of *Allium porrum* seed germination fifteen days after treatment with (a) extract of peat/sand substrate used to grow Wollemi Pine (left) and (b) extract of peat/sand not used to grow Wollemi Pine (right).

Figure 4.2 Comparison of *Sorghum bicolor* seed germination fifteen days after treatment with (a) extract of peat/sand substrate used to grow Wollemi Pine (left) and (b) extract of peat/sand not used to grow Wollemi Pine (right).

4.3.2 Effects of Extract pH on Seed Germination

The pH values of the Wollemi Pine soil extracts were not significantly different from those of the respective control extracts (all $P > 0.05$; data not shown). Linear regression analyses of leek seed germination data and treatment pH found that pH was not significantly correlated with seed germination after five days ($R^2 = 0.0428$, $P = 0.23$, $F = 1.48$, $df = 34$), ten days ($R^2 = 0.0333$, $P = 0.29$, $F = 1.14$, $df = 34$), and fifteen days ($R^2 = 0.0331$, $P = 0.30$, $F = 1.13$, $df = 34$) of experimentation. The same analysis performed on sorghum germination data five ($R^2 = 0.0005$, $P = 0.90$, $F = 0.02$, $df = 34$), ten ($R^2 = 0.0006$, $P = 0.89$, $F = 0.02$, $df = 35$), and fifteen days ($R^2 = 0.0015$, $P = 0.83$, $F = 0.05$, $df = 34$) following exposure to the treatments also revealed no significant relationship between pH and germination.

4.4 Discussion

Allelopathic potential provides plants with chemical mechanisms to restrict the growth of neighbouring plants, but there have been no previous reports demonstrating allelopathic potential in Wollemi Pine. Plants closely related to the Wollemi Pine, including *Araucaria cunninghamia* and *Podocarpus nagi*, have been reported to have allelopathic potential (Bevege, 1968; Ohmae et al., 1999), but the effects on target plants and the nature of the chemicals involved have not been fully explored. Allelopathic effects of other conifers such as members of the Pinaceae have also been investigated. For example, Wilt et al. (1993) reported high total monoterpene content in pinyon pine (*Pinus monophylla* Torr. & Frem.) needles, including the compound β-phellandrene, which may have phytotoxic

properties. Also, Kil and Yim (1983) demonstrated that allelochemicals released by red pine (*Pinus densiflora* Sieb. & Zucc.) needles and roots inhibited germination and growth for a host of understorey plants. These phytochemicals were later identified as 9α13β-epidioxyabeit-8(14)en-18-oic acid (Kato-Noguchi et al., 2009). Ponderosa pine (*Pinus ponderosa* Dougl.) also exhibits allelopathic activity, as demonstrated by the reduction in germination and seedling growth of *Andropogon gerardii* and *A. scoparius* following exposure to pine needle, bark, and soil extracts (Lodhi and Killingbeck, 1982).

The current study was conducted following preliminary evidence that the co-culture of leeks and Wollemi Pines resulted in adverse effects on the germination and growth of the leek seeds and plants (Chapter 2, section 2.3.2.2). The first hypothesis, that germination would be different between leek and sorghum, was accepted because the germination of leek seeds was inhibited following exposure to Wollemi Pine soil extracts while the germination of sorghum seeds was not. The second hypothesis was rejected, therefore the differences in leek and sorghum germination were not due to pH differences between the extract samples. Thus, the results from this study indicate that water-soluble extracts from Wollemi Pines suppress seed germination of leeks but not of sorghum. This finding confirms that the toxic potential of Wollemi Pine cannot be generalized for all plants.

Variations in plant responses to allelochemicals have been documented in a variety of ecosystems, including agricultural and forest environments. For example, a study on the allelopathic effects of eastern hemlock (*Tsuga canadensis* (L.) Carriere) by Ward and McCormick (1982) found that aqueous hemlock litter extracts inhibited hemlock seedling regeneration but did not affect oak seedling growth. Also, sweet potato root extracts have been shown to inhibit seed germination of several weed species but germination of morning

glory and eclipta was not affected by the extracts (Peterson and Harrison Jr., 1991). Additional plants shown to exhibit selective allelopathic effects include watermelon (*Citrullus vulgaris*) (Kushima et al., 1998), spotted knapweed (*Centaurea maculosa*) (Callaway et al, 2005), and China fir (*Cunninghamia lanceolata*) (Chou et al., 1989). Susceptibility is a key determinant in these differential responses, and often depends on the species involved, the concentration of the phytotoxins received by the target species, and the introduction of stressors or environmental changes (Hill et al., 2006). In addition, susceptibility can change in a population over time if plants evolve tolerance to the effects of the allelochemistry of other plants in the community, similar to developing resistance to synthetic herbicides (Duke et al., 2001; Callaway et al., 2005). The ecological consequences of different responses to allelochemicals have been largely unexplored, but likely include an important role in determining plant community structure. The biological impact of Wollemi Pine allelopathic activity is a good subject for further investigation.

Allelopathic influences on leeks by other plant species have not been described in the literature, but reductions in seedling growth and root development of sorghum have been observed following exposure to unknown allelochemicals released from sunflower (*Helianthus annuus* L.) (Schon and Einhellig, 1982) and taro (*Colocasia esculenta* (L.) Schott) (Pardales Jr. et al., 1992). Therefore, it is possible that the extracts used in the current study did not contain the same phytotoxins that have been shown to affect sorghum germination.

While leek (Alliaceae) and sorghum (Poaceae) are both monocotyledonous plants, some of their anatomical and physiological characteristics differ significantly. For example, leeks develop thickened bulb-like leaf bases from which the roots protrude, while sorghum

plants do not produce these specialized structures (Hopkins and Hüner, 2004). Also, sorghum plants exhibit C4 carbon fixation, in which their internal leaf structure is modified to support warmer climates and lower carbon dioxide levels inside the leaves (Hopkins and Hüner, 2004).

While the germination process was completed for sorghum seeds treated with Wollemi Pine soil extracts, water-soluble allelochemicals within the extracts may block part of the seed germination pathway in leeks to inhibit germination. Some studies have attributed allelopathic suppression of germination to a disruption of the metabolic pathways responsible for mobilizing nutritive reserves (Gniazdowska and Bogatek, 2005). For example, Troc et al. (2009) found that mustard seeds treated with allelochemicals extracted from sunflower leaves did not experience the same changes in fatty acid and flavonoid content as the un-treated seeds due to decreased metabolic activity. Further research is required to determine both the stage at which leek seed germination is blocked by *W. nobilis* allelochemicals and the mechanism by which this occurs.

The outcome of this study has ecological relevance for Wollemi Pines. It is difficult to assess the competitive advantage that may be incurred by the release of allelochemicals without a better knowledge of the target plants and chemicals that are involved. The remaining natural Wollemi Pine populations are found growing in a rainforest amid ferns, eucalypts, and coachwood (Jones et al., 1995). The habitat is moist and borders a creek (Jones et al., 1995), which permits the distribution of allelochemicals upon their release. Mobilizing the phytotoxins away from their own populations is beneficial as the chemicals may also be detrimental to Wollemi Pine health. For example, spontaneous dieback has been observed in Wollemi Pines growing in the center of greenhouse benches, while the trees

around the bench perimeter remain healthy (Schneider, Wollemi Pine North America, personal communication). More work is needed to explain this occurrence and how it relates to the interaction of Wollemi Pines with other vegetation in the wild.

The allelopathic activity of Wollemi Pines also has implications in mycorrhizal research. Leeks are commonly grown as bioassay plants to provide a local source of arbuscular mycorrhizal fungal inoculum (e.g. Bécard and Piché, 1989). The negative response of leeks to Wollemi Pine soil extracts indicates that living colonized leek seedlings are likely not a suitable inoculum source for *W. nobilis*, whereas using live seedlings of sorghum may be a better choice (e.g. Kennedy et al., 1999). The use of dead segments of roots from either plant for inoculation purposes may still be a viable option.

4.5 Conclusion

This study presents the first preliminary evidence of the production of allelochemicals by Wollemi Pines that are toxic to some plants. This work has shown that leek seed germination is adversely affected by phytotoxins released by Wollemi Pines into the soil, while sorghum seed germination is not. More work is required to reveal how the seed germination process in leeks is targeted by the allelochemicals. The findings of this study also raise the question of the ecological impact of Wollemi Pine allelopathic potential. The phytotoxins released by *W. nobilis* may provide a competitive advantage over some plants while also being potentially self-damaging. A better understanding of the allelopathic activity of Wollemi Pines will be fundamental in future studies on this endangered conifer.

4.6 References

Bécard G; Piché Y. (1989) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytologist. 112: 77-83

Bevege DI. (1968) Inhibition of seedling hoop pine (*Araucaria cunninghamia* Ait.) on forest soils by phytotoxic substances from the root zones of Pinus, Araucaria and Flindersia. Plant and Soil. 29: 263-273

Callaway RM; Ridenour WM; Laboski T; Weir T; Vivanco JM. (2005) Natural selection for resistance to the allelopathic effects of invasive plants. Journal of Ecology. 93: 576-583

Chou C-H; Chang S-J; Cheng C-M; Wang Y-C; Hsu F-H; Den W-H. (1989) The selective allelopathic interaction of a pasture-forest intercropping in Taiwan. II. Interaction between kikuyu grass and three hardwood plants. Plant and Soil 116: 207-215

Czarnota MA, Rimando AM, Weston LA. (2003) Evaluation of root exudates of seven sorghum accessions. Journal of Chemical Ecology. 29: 2073-2083

Duke SO, Scheffler BE, Dayan FE, Weston LA, Ota E. (2001) Strategies for using transgenes to produce allelopathic crops. Weed Technology. 15: 826-834

Einhellig FA; Rasmussen JA; Hejl AM; Souza IF. (1993) Effects of root exudate sorgoleone on photosynthesis. Journal of Chemical Ecology. 19: 369-375

Gallet C. (1994) Allelopathic potential in bilberry-spruce forests: influence of phenolic compounds on spruce seedlings. Journal of Chemical Ecology. 20: 1009-1024

Geil RD; Guinel FC. (2002) Effects of elevated substrate-ethylene on colonization of leek (*Allium porrum*) by the arbuscular mycorrhizal fungus *Glomus aggregatum*. Can. J. Bot. 80: 114-119

Gniazdowska A; Bogatek R. (2005) Allelopathic interactions between plants. Multi-site action of allelochemicals. Acta Physiologiae Plantarum. 27: 395-407

Hill EC, Ngiuajio M, Nair MG. (2006) Differential response of weeds and vegetable crops to aqueous extracts of hairy vetch and cowpea. HortScience. 41: 695-700

Hopkins WG; Hüner NPA. (2004) Introduction to Plant Physiology. 3rd Ed. Hoboken, New Jersey: John Wiley & Sons Inc.

Jones WG; Hill KD; Allen JM. (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. Telopea. 6: 173-176

Kato-Noguchi H; Fushimi Y; Shigemori H. (2009) An allelopathic substance in red pine needles (*Pinus densiflora*). Journal of Plant Physiology. 166: 442-446

Kennedy LJ; Stutz JC; Morton JB. (1999) *Glomus eburneum* and *G. luteum*, two new species of arbuscular mycorrhizal fungi, with emendation of *G. spurcum*. Mycologia. 91: 1083-1093

Kil BS; Yim YJ. (1983) Allelopathic effects of *Pinus densiflora* on undergrowth of red pine forest. Journal of Chemical Ecology. 9: 1135-1151

Kushima M; Kakuta H; Kosemura S; Yamamura S; Yamada K; Yokotani-Tomita K; Hasegawa K. (1998) An allelopathic substance exuded from germinating watermelon seeds. Plant Growth Regulation. 25: 1-4

Lill RE; Waid JS. (1975) Volatile phytotoxic substances formed by litter of *Pinus radiata*. New Zealand Journal of Forest Science. 5: 165-170

Lodhi MAK; Killingbeck KT. (1982) Effects of pine-produced chemicals on selected understory species in a *Pinus ponderosa* community. Journal of Chemical Ecology. 8: 275-283

Mahall BE; Callaway RM. (1992) Root communication mechanisms and intracommunity distributions of two Mojave desert shrubs. Ecology. 73: 2145-2151

Mallik AU; Newton PF. (1988) Inhibition of black spruce seedling growth by forest-floor substrates of central Newfoundland. Forest Ecology and Management. 23: 273-283

Offord C; Meagher P. (2006) Wollemi Pine: from the wild to the world. Chronica Horticulturae. 46: 10-13

Ohmae Y; Shibata K; Yamakura T. (1999) The plant growth inhibitor nagilactone does not work directly in a stabilized *Podocarpus nagi* forest. Journal of Chemical Ecology. 25: 969-984

Panasiuk O; Bills DD; Leather GR. (1986) Allelopathic influence of *Sorghum bicolor* on weeds during germination and early development of seedlings. Journal of Chemical Ecology. 12: 1533-1543

Pardales JR Jr.; Kono Y; Yamauchi A; Iijima M. (1992) Seminal root growth in sorghum (*Sorghum bicolor*) under allelopathic influences from residues of taro (*Colocasia esculenta*). Annals of Botany. 69: 493-496

Peterson JK, Harrison Jr. HF. (1991) Differential inhibition of seed germination by sweetpotato (*Ipomoea batatas*) root periderm extracts. Weed Science. 39: 119-123

Putnam AR. (1988) Allelochemicals from plants as herbicides. Weed Technology. 2: 510-519

Putnam AR, Defrank J, Barnes JP. (1983) Exploitation of allelopathy for weed control in annual and perennial cropping systems. Journal of Chemical Ecology. 9: 1001-1010

Rasmussen JA; Hejl AM; Einhellig FA; Thomas JA. (1992) Sorgoleone from root exudate inhibits mitochondrial functions. Journal of Chemical Ecology. 18: 197-207

Saito M; Stribley DP; Hepper CM. (1993) Succinate dehydrogenase activity of external and internal hyphae of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae* (Nicol. & Gerd.) Gerdemann and Trappe, during mycorrhizal colonization of roots of leek (*Allium porrum* L.), as revealed by in situ histochemical staining. Mycorrhiza. 4: 59-62

Schon MK; Einhellig FA. (1982) Allelopathic effects of cultivated sunflower on grain sorghum. Botanical Gazette. 143: 505-510

Singh HP; Kohli RK; Batish DR; Kaushal PS. (1999) Allelopathy of gymnospermous trees. J. For. Res. 4: 245-254

Troć M; Skoczowski A; Barańska M. (2009) The influence of sunflower and mustard leaf extracts on the germination of mustard seeds. Journal of Thermal Analysis and Calorimetry. 95: 727-730

Ward HA, McCormick LH. (1982) Eastern hemlock allelopathy. Forest Science. 28: 681-686

Weston LA. (1996) Utilization of allelopathy for weed management in agroecosystems. Agronomy Journal. 88: 860-866

Wilt FM; Miller GC; Everett RL; Hackett M. (1993) Monoterpene concentrations in fresh, senescent, and decaying foliage of single-leaf pinyon (*Pinus monophylla* Torr. & Frem.: Pinaceae) from the Western Great Basin. Journal of Chemical Ecology. 19: 185-194

Chapter 5: Discussion, Conclusion, Future Directions

Mycorrhizal colonization of *Wollemia nobilis* Jones, Hill & Allen (Wollemi Pine) has been largely unexplored to date. The single publication addressing this subject reports evidence of arbuscular mycorrhizas and *Wilcoxina*-like structures in adult Wollemi Pines growing in Wollemi National Park (McGee et al., 1999). To date, there are no published research studies exploring the role of mycorrhizal fungi in Wollemi Pine survival. This thesis describes the first reported mycorrhizal inoculation of Wollemi Pines in both laboratory and field environments.

Chapter 2 describes two inoculation experiments of Wollemi Pine using arbuscular mycorrhizal fungi and *Wilcoxina mikolae*. The major finding of these experiments was that colonization was not detected in any of the examined Wollemi Pine roots. In spite of this, there were significant differences in the nutrient composition and growth of inoculated plants compared to uninoculated controls. Also, fungal endophytes were detected in the root systems of all experimental plants. Another important result from this study was that the co-culture of leeks with inoculated Wollemi Pines resulted in death of all the leek plants and a complete lack of leek seed germination within the experimental pots. More research is required to determine whether Wollemi Pines are mycorrhizal and to understand the effects of the association. First, future studies should focus on developing effective inoculation methods and determining compatible fungal symbionts. Appropriate controls must be used in these studies to eliminate unnecessary experimental variables, such as the addition of nutrients in the substrate from the inoculum. The reasons for differences in tissue nutrient composition between inoculated and uninoculated plants may also be explored by measuring and manipulating the nutrient content within the growth substrate, and quantifying the

nutrients in the tissue after the experimental period. The ubiquitous presence of root endophytic fungi in Wollemi Pines also warrants further study. Attempts must be made to identify these fungi and evaluate their effects on Wollemi Pine health. The experiments presented in Chapter 2 provide fundamental results that supply important information for researchers in this field.

Chapter 3 presents a field study on Wollemi Pines inoculated in a ponderosa pine forest in Kelowna, British Columbia. Inoculum was present in the soil and on nearby grass roots, but was also provided to some plants as colonized leek roots or in potting mix containing fungal propagules. As in Chapter 2, mycorrhizal colonization was not detected in any of the Wollemi Pine roots that were examined. However, there were significant treatment differences in shoot tissue nutrient composition, but the lack of a proper control treatment creates difficulty in understanding the reason for these differences. As in Chapter 2, explaining the observed differences requires further study. This study was also the first to identify *Cylindrocarpon pauciseptatum* and *Phialocephala fortinii* as the fungal endophytes in Wollemi Pine roots, but more research is required to evaluate the nature of this symbiosis. This study provides an interesting report of temperate rainforest trees surviving and growing in non-native conditions, but it would be valuable to perform a similar experiment in Australia, where the environment and the fungal symbionts may be more suitable for the plants. To increase the chance of colonization, the length of the study and the age of the trees may both be increased. Similar to Chapter 2, although colonization was not detected, this study has advanced research in this field.

The first assessment of allelopathic potential in Wollemi Pine roots is described in Chapter 4. This study presents preliminary evidence of allelopathic chemicals produced by

Wollemi Pine roots that affect leek seed germination but do not affect sorghum seed germination. The effects of allelopathic interactions between Wollemi Pines and surrounding species is important to consider in conservation efforts, as outplanting young trees is likely to influence nearby vegetation. More investigation is required in this field, both to identify the chemical(s) responsible for the effects and to evaluate the effects of the chemical(s) on other plants. Determining target plant species susceptible to Wollemi Pine allelochemicals, as well as the biological processes of these plants that are affected by the chemicals, is an important step in this research area. Future studies may take a similar approach to the current study but instead use Australian plant species that naturally grow with Wollemi Pines. Allelopathy studies in the field may introduce additional variables, including the concentration of chemicals received by target plants or potential chemical degradation by microbes, that must also be addressed. This chapter presents the first evidence of allelopathic activity in Wollemi Pines, and the results are important to consider in future studies of this species.

The function of Wollemi Pine mycorrhizas must be fully understood prior to their use in conservation efforts. Studies of Wollemi Pine root interactions with fungal endophytes and with other plants are also required to gain a better understanding of this species. This thesis demonstrates the challenges in working with a rare and unique plant species such as *W. nobilis*, but has also provided a starting point from which future inoculation and allelopathy studies may be based.

5.1 References

McGee PA; Bullock S; Summerell BA. (1999) Structure of mycorrhizae of the Wollemi pine (*Wollemia nobilis*) and related Araucariaceae. Aust. J. Bot. 47: 85-95