PHYSIOLOGICAL EFFECTS OF INDIGENOUS ARBUSCULAR MYCORRHIZAL ASSOCIATIONS ON THE SCLEROPHYLL *AGATHOSMA BETULINA* (BERG.) PILLANS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signed: ……

Date: 26 December 2004

SUMMARY

The Mountain Fynbos biome, a division of the Cape Floristic Region (CFR), is home to round-leafed Buchu [*Agathosma betulina* (Berg.) Pillans], one of South Africa's best-known endangered herbal medicinal plants. *Agathosma betulina* is renowned as a traditional additive to brandy or tea, which is used for the treatment of a myriad of ailments. In its natural habitat, *A. betulina* thrives on mountain slopes in acid and highly leached gravelly soils, with a low base saturation and low concentrations of organic matter. To adapt to such adverse conditions, these plants have formed mutualistic symbioses with arbuscular mycorrhizal (AM) fungi. In this study, the effect of indigenous AM taxa on the physiology of *A. betulina* is investigated. In addition, the AM taxa responsible for these physiological responses in the plant were identified using morphological and molecular techniques.

Agathosma betulina was grown under glasshouse conditions in its native rhizosphere soil containing a mixed population of AM fungi. Control plants, grown in the absence of AM fungi, were included in the experimentation. In a time-course study, relative growth rate (RGR), phosphorus (P)-uptake, P utilization cost, and carbon (C)-economy of the AM symbiosis were calculated. The data showed that the initial stages of growth were characterized by a progressive increase in AM colonization. This resulted in an enhanced P-uptake in relation to non-AM plants once the symbiosis was established. Consequently, the lower P utilization cost in AM plants indicated that these plants were more

efficient in acquiring P than non-AM plants. When colonization levels peaked, AM plants had consistently higher growth respiration. This indicated that the symbiosis was resulting in a C-cost to the host plant, characterized by a lower RGR in AM plants compared to non-AM plants. Arbuscular mycorrhizal colonization decreased with increasing plant age that coincided with a decline in P-uptake and growth respiration, along with increases in RGR to a level equal to non-AM plants. Consequently, the AM benefit was only observed during the initial stages of growth. In order to identify the AM fungi *in planta*, morphological and molecular techniques were employed, which indicated colonization by AM fungi belonging to the genera *Acaulospora* and *Glomus.* Phylogenetic analyses of a dataset containing aligned 5.8S ribosomal RNA gene sequences from all families within the Glomeromycota, including sequences obtained during the study, supported the above mentioned identification.

OPSOMMING

Die Fynbos bergbioom, ‘n onderafdeling van die Kaapse Floristiese Streek, huisves rondeblaar Boegoe [*Agathosma betulina* (Berg.) Pillans], een van Suid Afrika se bekendste bedreigde medisinale plante. *Agathosma betulina* is bekend vir sy gebruik as tinktuur vir die behandeling van verskeie kwale. Die plant kom voor in bergagtige streke, in suur en mineraal-arm grond, met ‘n lae organiese inhoud. Gevolglik, om aan te pas by hierdie ongunstige kondisies, vorm die plante simbiotiese assosiasies met blaasagtige, struikvormige mikorrisa (BSM). In die huidige studie is die effek van hierdie BSM op die fisiologie van *A. betulina* ondersoek. Die identiteit van die BSM is ook gevolglik met morfologiese en molekulêre identifikasie tegnieke bepaal.

Agathosma betulina plante is onder glashuis kondisies in hul natuurlike grond gekweek, wat ‘n natuurlike populasie van BSM bevat het. Kontroles is ook in die eksperiment ingesluit en hierdie stel plante is met geen BSM geïnokuleer nie. Gevolglik is die relatiewe groeitempo, fosfor opname, fosfor verbuikerskoste asook die koolstof ekonomie van die plante bereken. Die data het getoon dat die eerste groeifase gekarakteriseer is deur toenames in BSM kolonisasie vlakke. Dit het tot ‘n hoër fosfor opname in BSM geïnokuleerde plante gelei. Die laer fosfor verbuikerskoste gedurende hierdie fase het aangedui dat die plante wat geïnokuleer is met BSM oor beter meganismes beskik het om fosfor uit die grond te bekom. Toe BSM kolonisasie vlakke gepiek het, was groei respirasie hoër in BSM geïnokuleerde plante as in die kontroles. Dit het aangedui dat die BSM

kolonisasie van plante tot hoër koolstof kostes vir hierdie plante gelei het, wat weerspieël is in die laer groeitempo van die BSM geïnokuleerde plante. Die BSM kolonisasie vlakke het gedaal met toenemende ouderdom van hul gasheer plante, wat gekarakteriseer is deur ‘n laer opname van fosfor en laer groei respirasie, tesame met ‘n toename in relatiewe groeitempo tot vlakke soortgelyk aan die van die kontrole plante. Die BSM voordele vir die plant is dus net gedurende die eerste groeifase waargeneem. Die BSM wat verantwoordelik is vir hierdie fisiologiese veranderinge is gevolglik geïdentifiseer met behulp van morfologiese en molekulêre tegnieke en dit is gevind dat BSM wat behoort tot die genera *Acaulospora* en *Glomus* binne hierdie plante voorkom. Filogenetiese analise gegrond op opgelynde 5.8S ribosomale RNA geen volgordes afkomstig van al die families binne Glomeromycota asook volgordes gevind in die studie, het die bogenoemde identifikasie gestaaf.

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CHAPTER 1: Introduction

INTRODUCTION

1.1. MOTIVATION

Agathosma betulina (Berg.) Pillans, also popularly known as round-leafed Buchu, is one of South Africa's best-known medicinal plants (Van Wyk *et al.* 1997; Coetzee *et al.* 1999). The plant is acclaimed for its use as blackcurrant flavoring in the food industry, but it also has pharmaceutical applications since it is used in the production of shampoos, mouthwashes, skin care products and insect repellents (Van Wyk *et al* 1997; Simpson 1998; Coezee *et al.* 1999; Lis-Bachin *et al.* 2001). In addition, *A. betulina* is used in perfumes and room fresheners, as well as in aromatic oils (Collins *et al.* 1996; Lubbe *et al.* 2003). Demographic trends in developed countries indicating a growing market for essential oils and the recent development of a Buchu fixative used in the cosmetics industry, have firmly established *A. betulina* as an agricultural crop in the Western Cape (Coetzee 2001). However, as is the case with many so-called common property resources that are not subject to defined ownership rights, this Fynbos plant has become vulnerable to over-exploitation in its natural habitat (Cunningham 1991).

Agathosma betulina is indigenous to the Mountain Fynbos biome of the south-western Cape, which forms part of the Cape Floristic Region, a Mediterranean climatic zone (Spreeth 1976; Moll *et al.* 1984). The floristically diverse Fynbos vegetation of this region has evolved, amongst others, in response to frequent stochastic fires and leached soils with a low nutrient status (Allsopp and Stock

1993; Linder 2003). As the majority of nutrients are only released during the short post-fire period, seedling establishment following wild fires in Fynbos is a period where efficient uptake of nutrients is particularly critical. Therefore, it was postulated that acquisition of nutrients from the soil in competition with other plants and organisms would best be mediated by arbuscular mycorrhizal (AM) fungi (Allsopp and Stock 1993). However, when exposed to increases in nutrient supply rate, the growth responses of wild plants from low nutrient environments are orders of magnitude lower than those of plants adapted to nutrient rich soils (Chapin 1988). Hence, it was suggested that AM fungi are less beneficial to slow growing wild plants from low nutrient environments than to rapidly growing crop plants (St John and Coleman 1983; Koide *et al.* 1988; Koide 1991). The general assumption is that plants with low, inflexible growth rates, high nutrient reallocation, and low tissue turnover will make low demands on their environment for nutrients such as phosphorus (P), the uptake of which is usually enhanced by AM fungi. However, as the availability of soil P is often transitory, a slow uptake mechanism, to complement the low requirement over time, would be disadvantageous as wild plants rely on acquiring nutrients rapidly, in excess of immediate needs, during periods of availability (Chapin 1988).

In general, the association between AM fungus and host plant may be considered as mutualistic (Allen 1991). The host plant receives mineral nutrients from outside the root's depletion zone via the extraradical fungal mycelium, while the heterotrophic mycobiont obtains photosynthetically produced carbon (C) compounds from the host. The C-cost of the fungus may be considerable, with

the fungus receiving up to 23% of the plant's photosynthetically fixed C (Kucey and Paul 1982; Snellgrove *et al.* 1982; Koch and Johnson 1984; Jakobsen and Rosendahl 1990). Therefore, enhanced nutrient uptake of AM colonized Fynbos plants, such as *A. betulina*, may be balanced by the cost of the symbiosis, in terms of the C supplied by the plant.

We are interested in the interactions of AM fungi with woody, slerophyllous *A. betulina* plants during seedling establishment in nutrient poor soils. The aim of this study was thus to investigate the effects of indigenous AM fungi on growth and P nutrition of *A. betulina*, as well as the C-economy of the symbiosis during AM development. As the identity of the indigenous AM fungi colonizing the roots of *A. betulina* is currently obscure, the study also aimed to use morphological and molecular methods to identify the AM taxa actively colonizing the roots of *A. betulina*.

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CHAPTER 2: Literature review

LITERATURE REVIEW

2.1. *AGATHOSMA BETULINA* (BERG.) PILLANS (SYN. BAROSMA BETULINA) - AN INDIGENOUS MEDICINAL PLANT RESOURCE OF SOUTH AFRICA

South Africa is blessed with a rich biodiversity and more than 22,000 plant species occur within its boundaries (Germishuizen and Meyer 2003). Despite the enormous richness in plant species, relatively few of these plants are economically utilized (Van Wyk *et al.* 1997). However, business ventures that have evolved from the use of indigenous plants are the trade in medicinal and cultural plants, food crops and ornamental plants. In addition, indigenous medicinal plants are used by more than 60% of South Africans in their health care needs or cultural practices. One of South Africa's best-known herbal medicinal plants, recognized in the international trade, is *Agathosma betulina* (Berg.) Pillans (Coetzee *et al.* 1999).

2.1.1. Botanical description: The name "agathosma" was derived from the Greek words "agathos", and "osma", which means "good" and "smell" respectively (Van Wyk and Gericke 2000). *Agathosma betulina* belongs to the family Rutaceae and is popularly known as round-leafed Buchu (Spreeth 1976; Lubbe *et al.* 2003). The plant is a perennial shrub that reaches two meters in height (Spreeth 1976; Van Wyk *et al.* 1997). This odiferous, multi-stemmed bush has twiggy, somewhat angular branches of a purplish-brown color (Spreeth 1976). During the winter months, the plant displays small, star-shaped white to

pale purple flowers (Fig. 2.1), making it sought after in the ornamental industry (Spreeth 1976; Coetzee *et al.* 1999). The coriaceous leaves occur opposite each other and are almost sessile with a very short petiole (Spreeth 1976). The leaves are about 20 mm long, obovate and wedge-shaped toward the petiole, with a rounded apex, which curves backwards. The fruit comprises of five upright carpels, each containing a single, oblong and shining black pyriform seed. The surface of the leaves is nearly free from trichomes, while the margins and lower surfaces are bordered with sharp serratures and conspicuously marked with oil glands appearing as pellucid spots set at the base of each serrature. A characteristic trait of all the species in the genus is the distinguishing strong, aromatic mint-like smell caused by volatile oils secreted by idioblasts on the glandular epidermis of the leaves (Van Rooyen *et al.* 1999). Compounds pertaining to the leaves of this plant include flavonoids (rutin, diosmin and quercitin), vitamins of the B group, tannins and mucilage, as well as 1.7% to 2.5% volatile oil, resulting in the plant being utilized as a source of essential oils (Bruneton 1995).

2.1.2. Essential oils of *A. betulina*: Different *Agathosma* species have different chemical characteristics and proportions of desirable oil components (Collins *et al.* 1996; Posthumus *et al.* 1996). Steam-distilled essential oils of *A. betulina* predominantly contain diosphenol and isomenthone, as well as limonene, pulegone, menthone, mercaptonmenthone and acetylthio-menthone. In addition, essential oils of this plant also contain relatively low amounts (<3%)

of 8-mercapto-p-menthan-3-one. Its pungency and fragrance are important for imparting a blackcurrant flavor to the oil, which is used in flavoring blackcurrant food products (Coetzee *et al.* 1999). It is also used in the perfume industry, as a constituent of beauty/cosmetic products, as well as in the pharmaceutical industry (Collins *et al.* 1996; Lis-Bachin *et al.* 2001; Lubbe *et al.* 2003). The demand for *A. betulina* is much higher than that of the closely related *A. crenulata* due to the larger content in desirable oil components of the former, especially diosphenol and 8-mercapto-p-menthan-3-one (Collins *et al.* 1996). In addition, the oil of *A. betulina* contains a lower pulegone concentration, which is a potentially toxic component (Van Wyk and Gericke 2000). Other uses for the essential oils of *A. betulina* are for medicinal purposes, mainly due to their antiseptic and diuretic properties (Bruneton 1995).

2.1.3. Medicinal and cultural uses: Regardless of its popularity in the essential oil industry, *A. betulina* also has a great reputation as a phytomedicine (Coetzee *et al.* 1999). Undoubtedly, much of the knowledge acquired on the medicinal values of this plant stems from the traditions of this country's earliest human inhabitants when necessity, trial and error led to discoveries of its curative properties (Van Wyk *et al.* 1997). As part of the cultural heritage of the San and Khoi pastoralists, a decoction of the leaves were traditionally employed to anoint the body (after mixing the powdered, dried leaves with sheep fat) probably both for cosmetic reasons and as an antibiotic. For medicinal use, the leaves were chewed to relieve stomach complaints, while the roots were used to treat

snakebites. Dutch colonists quickly followed suit and *A. betulina* became a popular and famous Cape medicine. The leaves were steeped in brandy and this tincture (commonly known as Buchu brandy) was an everyday remedy for stomach problems along with the treatment of minor digestive disturbances.

A few years after Jan van Riebeeck set foot in Africa, *A. betulina* undertook its first journey to Europe (Bradley 1992). Bales of *A. betulina* leaves were even listed on the cargo manifest of the *Titanic* on its doomed voyage across the Atlantic! Buchu vinegar, in particular, played an important role in the Crimean War and World War I as a powerful antiseptic to clean wounds. In 1821, *A. betulina* was officially registered as a medicine and introduced into conventional medicine by a London drug firm, Reece & Co., as a remedy for cystitis urethritis, nephritis and catarrh of the bladder, as well as for gout.

Agathosma betulina has subsequently enjoyed an unbroken reputation as one of the Cape Floral Kingdom's most robust natural elixirs and its products (Fig. 2.2a, b) were still widely used as a traditional South African household medicine for the treatment of a myriad other ailments, including for the treatment of cholera, reduction of inflammation of the colon, gums and sinuses, the symptomatic relief of rheumatism and high blood pressure, for strained muscles and even as a tonic for horses (Van Wyk *et al.* 1997; Coetzee *et al.* 1999; Van Wyk and Gericke 2000).

Today, it is principally employed as supportive treatment in chronic diseases of the urino-genital organs, as in cases of chronic inflammation of the mucous membrane of the bladder, irritable conditions of the urethra, in urinary muco-

purulent discharges with increased deposit of uric acid and in incontinence connected with diseased prostate (Bruneton 1995). An infusion of *A. betulina* is then taken as a diuretic and diaphoretic, since it is thought to be a mild urinary antiseptic (Van Wyk *et al.* 1997; Van Wyk and Gericke 2000).

2.1.4. Economic exploitation: As the renewed worldwide popularity of phyto-medicines as safe, natural alternatives to chemical drugs has grown, many opportunities for crop development and acclimatization of *A. betulina* in South Africa have been created (Coetzee *et al.* 1999). However, no major developments can be expected as long as there are no sustainable supplies of high quality raw material from which the various *A. betulina* products are made. Prices are mostly driven by exports to Europe, where the highest demand is met by the food industry, with an estimated value of R20 million per annum (WESGRO 1999). At present, 1 kg of fresh material costs between R40 and R60 (R5.71=US$ 1) and the price of 1 kg of dry leaves is *ca.* R320. Oil is obtained from fresh material with a yield of 1 or 2%, and its market price reaches R5,500-6,000/kg. However, international demand for natural flavors and fragrances is expected to rise by over 7% per year in the next decade (WESGRO 2000). As a consequence of the high prices, over-harvesting and illegal poaching form the main threat to this commercially exploited medicinal plant (Cunningham 1991). Concern has been raised about the conservation status of the species, danger of extinction in the wild being the worst scenario. Therefore, cultivation of *A.*

betulina seems the only viable alternative to service the ever-increasing demand for the product and to safeguard the species in the wild (Blomerus 2002).

2.1.5. Natural habitat: *Agathosma betulina* is a Fynbos sclerophyll and has a restricted natural distribution area in the pristine, Mountain Fynbos biome, confining it to the south-western portion of the Cape of Good Hope, South Africa (Collins *et al.* 1996; Van Wyk *et al.* 1997). The biome is characterized by two pronounced gradients common to all climatic elements; a North-South gradient from the great escarpment (32°S) to the southern coast (34°S) and a West-East gradient from the West coast (18°E) to the South-East coast at 28°E (Day *et al.* 1979). A Mediterranean-type climate prevails in the biome, which has an annual rainfall that ranges from 400 to 700 mm or more, and mean annual temperatures throughout the region close to 17°C.

Agathosma betulina occurs on mountain slopes at an altitude of between 102 and 203 m above sea level, often in small clusters of individuals with variable density (Spreeth 1976; Van Rooyen *et al.* 1999). This patchy pattern of distribution is characteristic of plants growing in semi-arid to arid environments, including Fynbos (Allen *et al.* 1995).

Fynbos is the most important type of heathland found in South Africa (Specht 1979) and is the term used to describe the characteristic vegetation of a well-defined landscape of the south-western and southern Cape Province (Kruger 1979). The word “heath”, was derived from the Germanic word “heide”, meaning “uncultivated stretch of land”, or “waste land” (Specht 1979). The Fynbos

vegetation type is one of several distinctive vegetation types occurring in the Cape Floristic Region (CFR) (Goldblatt and Manning 2000). The CFR has been described as the richest and smallest of the world's six floral kingdoms, covering only 0.04% of the earth's surface (Hall 1978). This region supports more than 9000 indigenous vascular plants (Goldblatt and Manning 2000), which have evolved, amongst others, in response to leached soils of amongst the lowest nutrient status worldwide (Allsopp and Stock 1993a; Linder 2003). These soils are especially nutrient poor in the mountainous regions (Fry 1987).

2.1.6. Chemical characteristics of soil: Mountain Fynbos soils, the natural soils of *A. betulina*, are mainly podzolics derived from quartzites and are coarse to medium textured sands (Spreeth 1976; Kruger 1979). These acidic soil types, ranging from pH 3.5-5.5, have been described as oligotrophic in being strongly leached, with base saturation levels of around 20 to 40 % (ratio of net exchangeable cation to cation exchange capacity) (Kruger 1979; Specht and Moll 1983). It was found that quartzite-derived soil organic carbon content (C_T) values were approximately 1%, while the nitrogen (N) concentration was no higher than 0.06% (Fry 1987). The presence of highly recalcitrant organic compounds combined with low microbial activity may be responsible for the slow rate of N mineralization and hence the low availability of N (DeBano and Dunn 1982). Nitrogen is also lost through denitrification (Rundel *et al.* 1983), and through particulate loss or volatilization as a result of fire (DeBano and Dunn 1982). In

addition to N deficiency, these soils also contain negligible amounts (3 – 40 μg/g) of available phosphorus (P) (Kruger 1979).

2.1.7. Carbon as an abundant resource: Nutrient-poor systems such as Fynbos are often rich in C (Stock *et al.* 1992). This surplus C in the system is not allocated to vegetative plant tissue development and maintenance, but rather utilized in an array of plant secondary compounds and mechanical structures. These mechanical structures have profound influences upon abiotic and biotic processes in Fynbos ecosystems. An example of one such plant structural modification is the C-rich leathery, or so called sclerophyllous leaves.

2.2. FUNCTIONAL SIGNIFICANCE OF SCLEROPHYLLY IN FYNBOS

The Fynbos biome is exceptionally rich in flora occurring as sclerophyllous shrublands and heathlands (Day *et al.* 1979), which is characterized by the dominance of the sclerophyllous leaf (Stock and Allsopp 1992). Evergreen shrubs possessing sclerophyllous leaves occur in many ecosystems from the tropics to the poles, but are especially dominant in the five Mediterranean-type ecosystems of the world (Stock *et al.* 1992). Moreover, the biochemical basis and functional significance of this leaf type is uncertain (Stock and Allsopp 1992).

It has been suggested that a suite of interrelated leaf characteristics, including sclerophylly, is favoured in low nutrient environments as it entails an increased C return per unit of nutrient invested by the plant (Stock *et al.* 1992). It was also found that, for species in Mediterranean regions, a strong positive correlation

exists between leaf N and P, between leaf N and calcium (Ca) as well as between the index of sclerophylly and leaf P (Specht and Rundel 1990). Functional interpretations of sclerophylly have emphasized, firstly, its suitability as an adaptation to drought (Poole and Miller 1975); secondly, that it is an adaptation improving the efficiency of nutrient utilization in low nutrient environments (Specht and Rundel 1990); and thirdly, that this modification is a highly successful means of reducing herbivory (Chabot and Hicks 1982), because of its high fibre and low water content and thick cuticles (Stock *et al.* 1992). Fibre, wax and cutin are poorly digested by mammals, and high concentrations of these substances dilute the essential nutrients and energy contained within this leaf type.

2.3. FIRE AND ITS CENTRAL ROLE IN FYNBOS NUTRIENT CYCLING

The incidence of frequent fires rather than a Mediterranean-type climate, is the key environmental factor that is coupled with nutrient paucity (Stock and Allsopp 1992). High concentrations of tannins, resins and essential oils in the sclerophyllous leaves of many heathland plants increase the flammability of the community during periods of water stress (Specht 1979). Fires are a regular disturbance initiating successional changes, during which time alterations occur in the patterns of resource availability occur (Stock and Allsopp 1992). These changes reportedly follow definite patterns, with one school of thought suggesting that the availability of all resources (water, light and nutrients) is

elevated at the soil surface shortly after the disturbance, and that the availability of these resources diminishes with time.

Fire appears to be the major mineralizing agent in Fynbos, returning mineral elements held in the above ground phytomass and litter to the soil (Brown and Mitchell 1986). Firstly, post-fire nutrient flushes appear to be a characteristic of Fynbos systems and increased availability of N, P and cations has been reported (Musil and Midgley 1990). However, the availability of these elements appears to decrease rapidly to the low pre-fire levels (nine months for N) as elements become incorporated into plant biomass, as well as being immobilized by decomposer organisms.

Like N, P availability is enhanced during the early post-fire period (Brown and Mitchell 1986), but also rapidly (within four months) returned to pre-fire levels as it is immobilized in the soil flora or else sequestered in the above ground phytomass, rendering P largely unavailable (Chapman *et al.* 1989).

With the above as background, it is evident that fires do not merely initiate phases of regeneration, as in so many vegetation types, but are vital for the persistence of the Fynbos flora and vegetation in the CFR, as nutrients are only released in abundance during the short post-fire periods (Stock and Allsopp 1992). Concurrently, this fire-prone or pyrophylic vegetation is dominated by plants with life strategies tuned to this fire regime.

2.4. POST-FIRE SEEDLING ESTABLISHMENT

Plants typical of resource limited habitats (shaded, arid or low nutrient environments) are generally unable to acquire sufficient resources to support rapid growth and typically have low maximum potential growth rates (Chapin 1980). Concurrently, low growth rates are associated with P storage in low nutrient environments, because this allows the plant to take advantage of pulsed or unpredictable flushes in available nutrients (Chapin 1988). This ensures an adequate supply of P to support growth during periods when nutrients are generally unavailable. As the availability of soil P is often transitory, wild plants rely on acquiring nutrients rapidly to complement the low availability over time. Therefore, seedling establishment, following wild fires in Fynbos, is a period where efficient uptake of nutrients is particularly critical and acquisition of nutrients from the soil in competition with other plants and organisms would best be mediated by arbuscular mycorrhizal (AM) fungi (Allsopp and Stock 1993b). However, little is known about both the AM associations of plants in the CFR and their functional role in low nutrient ecosystems.

2.5. THE ARBUSCULAR MYCORRHIZAL FUNGI

Since their colonization of terrestrial ecosystems, plants have developed numerous strategies to cope with the diverse biotic and abiotic challenges that are a consequence of their sedentary life cycle (Gianinazzi-Pearson 1984). One of the most successful strategies is the ability of root systems to establish mutualistic symbiotic associations with microorganisms. Microbial activity in the

rhizosphere is a major factor that determines the availability of nutrients to plants and has a significant influence on plant health and productivity (Jeffries *et al.* 2003). Healthy terrestrial ecosystems are characterized by the presence of a diverse population of microorganisms (Linderman 1992). These microorganisms are generally concentrated in litter layers or the rhizosphere. The rhizosphere or root-soil interface is a complex system with multifarious interactions between microorganisms and the plant (Kleeber *et al.* 1983). "Rhizo" or "Rhiza" was derived from the Greek word meaning "root", but "sphere" has many meanings (Starkey 1958). The rhizosphere constitutes the rhizosphere soil (the volume of soil adjacent to and influenced by the root) and the root surface or rhizoplane, which includes the cells of the root cortex where invasion and colonization by endophytic microorganisms occurs (Jeffries *et al.* 2003).

An important and most frequent component of these endophytic microorganisms are soil fungi, some of which form mutualistic associations with plant roots, termed mycorrhizae (Linderman 1992). Several criteria can be used to distinguish mycorrhizae from other plant-fungus associations (Allen 1991). One is the mutualistic nature of the interaction, which is characterized by the flow of inorganic components from the fungus to the plant and organic components from the plant to the fungus. The other is the structural nature of the interaction in that the fungus extends both into the host plant and into the surrounding substrate.

Two morphological types of mycorrhizae exist, namely endomycorrhizae and ectomycorrhizae (Smith and Read 1997). Other types have been described

subsequently, but common practice delineated ecto- (those with the fungus outside the plant cells), ectendo- (those wherein the fungus penetrates the cortical cells but also forms a mantle surrounding the root) and endomycorhizae (those with hyphae penetrating the cell walls but lacking a mantle).

A biotrophic endomycorrhiza, the arbuscular mycorrhiza, is the most widespread plant-fungus symbiosis on earth, formed between over 80% of all families of land plants and a small group of common soilborne fungi belonging to the phylum Glomeromycota (Allen 1991). The fungi that are responsible for this symbiosis are known as the AM fungi as they produce characteristic finely branched tree-shaped, short-lived hyphal structures, termed arbuscules, inside the cortical cells of plant roots (Douds and Millner 1999). Earlier, the name vesicular-arbuscular mycorrhizal (VAM) fungi was used, but since not all fungi in the group produce vesicles, the term AM fungi is preferred (Morton and Benny 1990).

Considering the above mentioned interdependence between AM fungi and plants, it is not surprizing that evidence exists for the co-evolution of plants and these fungi (Brundrett 2002). Recent fossil evidence supports the existence of AM fungi in the earliest vascular land plants (*Aglaophyton*) that lived more than 400 million years ago in the early Devonian period. In addition, molecular phylogenetic research indicates that the most primitive AM fungi diverged from a closely related non-mycorrhizal taxon at about the same time (462-353 million years ago). The long co-evolutionary history of plants and AM fungi as proved by

these findings probably explains the extensive global distribution of AM fungi (Taylor *et al.* 1995).

2.6. ARBUSCULAR MYCORRHIZAL FUNGI: HABITAT

Arbuscular mycorrhizal fungi are ubiquitous in most natural ecosystems (Allen 1991). These include ecosystems ranging from the aquatic to deserts, from lowland tropical rain forests to forests at high latitudes and to canopy epiphytes. In addition, these fungi are also found in agro-ecosystems (Smith and Read 1997).

Several early reports suggested that AM fungi are primarily distributed vertically near the surface of most soils where labile nutrients were being released as a result of fire (Brown and Mitchell 1986) or from newly decomposing organic matter (Allen 1991). However, there is much experimental evidence indicating that AM colonization and occurrence are reduced in plants or soils with high concentrations of certain nutrients, especially P (Jakobsen 1986). In addition, AM fungi may be found up to 4 m deep into the soil profile (Allen 1991). These AM fungi are then heavily dependent on the frequency of passing roots to grow.

AM communities may vary greatly in different soil environments (Clark 1997). One of the principal factors affecting AM distribution and activity in soil is pH, as the activity of some AM taxa might be limited in acidic soils. However, it is known that plant roots can become colonized with AM fungi in soils with pH values as low as 2.7, and are frequently found at soil pH levels ranging from 3.64 to 3.70 which is characteristic of some Fynbos soils (Specht and Moll 1983).

2.7. ARBUSCULAR MYCORRHIZAL FUNGI IN FYNBOS

Although the vast majority of plants growing in natural ecosystems, including Fynbos, have not had their AM status confirmed (Allsopp and Stock 1993a), it is generally accepted that most terrestrial plants form AM associations (Smith and Read 1997). The AM status of plants reflects the taxonomic affinities and ecology of both the plants and the fungi (Read 1991).

Surveys of the mycorrhizal status of plants growing in the CFR revealed that 62% of indigenous sclerophyllous shrubs, mainly the shallow rooted Fynbos taxa such as the Rutaceae, generally form mutualistic associations with AM fungi (Allsopp and Stock 1993a). However, a previous study showed that AM colonization levels of established Fynbos vegetation are usually low (37%) (Allsopp and Stock 1994). Factors contributing to the low AM colonization may be the patchy distribution of AM infectivity in these soils, disturbances such as fire, or that AM colonization of roots is restricted to the first phase of the growth period, where efficient uptake of nutrients during the post-fire period is particularly important. Soil inoculum levels may thus limit initial colonization, but once colonization occurs, roots rapidly become mycorrhizal.

2.8. THE PROCESS OF AM COLONIZATION

In most cases, the anatomical and cytological changes induced by AM fungi colonizing the host do not induce root alterations recognizable with the naked eye (Bonfante-Fasolo 1984). Only in plants such as onions or other Liliaceae and maize, can AM roots be recognized by their yellow color. Another

characteristic, which can sometimes be an indication whether AM fungi are present, is the size and morphology of the roots, and in particular the development of root hairs. The observation that plant species that lack a fine root system and prolific root hair development are frequently more colonized and more dependent on AM fungi, could reflect the fact that the symbiotic fungus replaces certain root hair functions, such as mineral nutrient absorption.

AM colonization occurs solely in the epidermis and the cortical parenchyma of roots (Brundrett *et al.* 1996). The colonization process develops in stages, including an extramatrical phase with extramatrical hyphae and spores scattered in the surrounding soil, and an intraradical phase with unbranched intracellular hyphae, intercellular hyphae, branched intracellular hyphae (arbuscules) and vesicles developing.

2.8.1. Extramatrical phase, mycelium: Probably the most important feature of the arbuscular mycorrhiza and the most neglected in physiological and ecological research, is the extramatrical hyphal matrix (Allen 1991). The development and spread of the extramatrical phase of AM fungi differs greatly according to the type of soil, plant and fungus. In some cases the length of the AM hyphae may be 80 to 134 times more than that of the subtending mycorrhizal root (Tissdal and Oades 1979). In other cases, the hyphae appear to be less developed.

Morphologically, the extramatrical mycelium is continuous with the intraradical one, thus forming one infection unit (Allen 1991). Its growth usually results in fan-shaped mycelia consisting of dichotomously branched hyphae with few,

adventitious septa, radiating out from the extramatrical hyphae termed trunk or “runner” hyphae.

2.8.2. Extramatrical phase, hyphae: AM fungi have two distinct types of extramatrical hyphae, the “runner” hyphae and the absorbing hyphae (Allen 1991). The runner hyphae are thick-walled, larger hyphae that track roots into the soil or, in some cases, simply grow through the soil in search of additional roots. The hyphae that penetrate roots are initiated from runner hyphae. The absorbing hyphae also develop from the runner hyphae and form a dichotomously branching hyphal network extending into the soil from the runner hyphae. These hyphae appear to be the component of the fungus that absorbs nutrients from the soil for transport to the host. Clustered swellings characteristic of certain AM taxa, termed auxiliary bodies (external vesicles) may form on the extramatrical hyphae (Brundrett *et al.* 1996). In addition, AM spores are regularly associated with the extramatrical mycelial hyphae.

2.8.3. Extramatrical phase, asexual spores: AM fungi form external vesicle-like, globose to obovate spores to escape environmental stresses (Allen 1991). The spores are formed in the soil either singly and borne directly on extramatrical fertile hyphae (Fig. 2.3), in groups within a fruiting structure termed a sporocarp, or in loose or tight masses (from one to several hundred) within a hyphal matrix (Fig. 2.4). Arbuscular mycorrhizal spores (Fig. 2.3) germinate through the lumen of the subtending hyphae, depending on the AM species, while germination

structures (germ tubes) are synthesized from re-growth of the innermost layer of the spore wall (Brundrett *et al.* 1996). Some AM taxa may even form spores inside root cells (Douds and Schenck 1990). The thick-walled AM spores, with a dense cytoplasmic content rich in oil globules, vary in size from 15 to 20 μm in diameter and are usually attached to a subtending hypha (Brundrett *et al.* 1996). With age, the spores become vacuolated. Spores function as storage structures, resting stages and propagules. Depending on size, vertical distribution and the environmental characteristics, these structures may be dispersed by wind, water or animals, or they may simply remain quiescent in the soil until conditions adequate for growth are present or contact with a root is established (Allen 1991).

2.8.4. Intraradical phase: When in contact with a susceptible root, the extramatrical hypha swells apically and increases in size forming a more or less pronounced structure termed an appressorium (Gianinazzi-Pearson *et al.* 1980). The extramatrical mycelium may give rise to a number of entry points into the root (Allen 1991). Different ways of root penetration may occur, depending most probably on the wall-thickening pattern of the outer cells. The colonization hypha originating from the appressorium may directly penetrate the wall of an epidermal or exodermal (in older roots) cell and then enter the first intact layer of the cortical cells. The mechanism by which this occurs is not yet fully understood, but it was suggested that mechanical and/or enzymatic actions may play a role (Scannerini and Bonfante-Fasolo 1983). It then spreads intercellularly from the entry point

(Gianinazzi-Pearson *et al.* 1981). When the fungus is inside the root, the way in which it spreads varies, depending on the plant and fungus involved.

Although the fungus penetrates the cell wall and gives rise to the so called internal structures, it does not penetrate the cell membrane (Allen 1991). However, the surface area of the plasmalemma is dramatically increased in a mycorrhizal plant cell. This results in a substantial increase in surface area for absorption of nutrients compared with the absorptive area of a non-AM cell.

The internal structures are the organs wherein nutrients and C are being exchanged between the host and endophyte, and are probably the best-described components of the arbuscular mycorrhiza (Allen 1991). They also form the basis of the original distinctions between ecto- and endomycorrhizae.

2.8.5. Intraradical phase, intracellular hyphae: The outer cortical root layers are often colonized by internal structures called intracellular *Paris*-type hyphae, characterized by a linear or more often a looped arrangement, without any signs of branching (Brundrett *et al.* 1996). The colonization hypha of the AM fungus can form intracellular coils in the first cell to be infected, with similar coils being formed in neighboring cells. Alternatively, the colonization hypha penetrates the first cell without coiling and becomes organized in coils only in neighboring cells. According to Abbott (1982), the size of the intracellular unbranched hyphae depends on the type of fungus involved, while the host probably influences the number and the behavior of the hyphae.

During the cell-to-cell passage from the outer cortical layers to the inner ones, the host cell plasmalemma appears to be continuous (Brundrett *et al.* 1996). This suggests a rather complex sequence of events in the development of these structures. As the intracellular fungus reaches the periphery of the cell, the host plasmalemma, which is invaginated around the fungus and limiting the interfacial zone, becomes continuous with and adheres to the host cell wall. The fungus passes through the middle lamella and primary wall of the host, that has become continuous with the matrix material, probably by both a mechanical and an enzymatic mechanism. The fungus thus penetrates the underlying cell wall and ends up causing the invagination of the plasmalemma in the next cell. On reaching the middle area of the cortical parenchyma the fungus becomes intercellular due to a mechanism resembling the one discussed above. The colonization subsequently spreads along the root by intercellular hyphae running parallel to the root axis.

2.8.6. Intraradical phase, intercellular hyphae: Intercellular *Arum*-type hyphae produced by coils or directly by penetrating hyphal branches, are usually found in the intermediate layers of the cortical parenchyma (Brundrett *et al.* 1996). These hyphae dilate the intercellular spaces and sometimes occur in bundles consisting of three or four individual hyphae. They run in the cortical parenchyma for considerable distances (up to several millimeters) and sometimes have a wavy form as they follow the outline of the host plant cells.

They often have intermittent projections and are at times swollen. These hyphae also give rise to arbuscules.

2.8.7. Intraradical phase, arbuscules: In the inner layers of the cortical parenchyma, intercellular hyphae penetrate the cortical cells giving rise to a complex hyphal branching system, similar to “small bushes”, which are called arbuscules (Brundrett *et al.* 1996). The arbuscule is the most significant structure in the AM complex, in particular from a functional viewpoint, for the arbuscule is thought to be the preferential site for fungus/plant metabolite exchanges (Scannerini and Bonfante-Fasolo 1983). The presence of arbuscules within a root is generally considered as a sign of a functional symbiosis (Regvar *et al.* 2003). The arbuscule trunk resembles the intercellular hypha, from which it proliferated, in size and bifurcates repeatedly inside the cell, thus giving rise to smaller branches (Brundrett *et al.* 1996). The latter proliferate onto smaller branched hyphae with short bifurcate terminals.

Transmission electron microscopy shows that these smaller hyphae of the arbuscule, which are always surrounded by the invaginated host plasmalemma, contain numerous nuclei, mitochondria, glycogen particles, lipid globules, abundant polyvesicular bodies and electron dense granules inside small vacuoles (Bonfante 1994). These vacuoles are of great significance, for energy dispersive X-ray analysis has shown the presence of high levels of P and Ca within the electron-dense granules. Consequently, they are thought to be rich in polyphosphates. Furthermore, they are the sites of intense alkaline phosphatase

and possibly adenosine triphosphate (ATP)ase activities. In the thinner branches, the vacuoles become dominant and the electron-dense granules disappear.

The arbuscule life span is limited to a few days, four or five, after which the smaller arbuscular branches show disorganized cytoplasmic contents, loss of membrane integrity, and finally appear as an amorphous mass (Bonfante-Fasolo 1978). The walls of the empty zones collapse and then aggregate into clumps. Collapsed arbuscules, including large arbuscular clumps formed by the aggregation of smaller ones filling the host cell, are often observed in roots collected from the field. Another structure often observed in abundance in these roots are vesicles (Bonfante-Fasolo 1984).

2.8.8. Intraradical phase, vesicles: Vesicle development is initiated soon after the first arbuscules appear, but continues when the arbuscules senesce (Brundrett *et al.* 1996). Vesicles are globose bodies caused by an intercalary or terminal swelling of the AM hypha. Vesicles (30 to 50 μm or 80 to 100 μm in diameter) found within roots can be intercellular or intracellular, and may be found in both the inner and the outer layers of the cortical parenchyma. Intercellular vesicles and host walls are in direct contact, while intracellular vesicles are usually enclosed by a layer of condensed host cytoplasm (Scannerini and Bonfante-Fasolo 1983). The outer vesicle surface appears smooth without ornamentation, while some may form lobed or irregular intracellular vesicles. The walls appear to be trilaminate, constituting of layers of

varying electron density (Bonfante 1994). The cytological organization of the vesicles (mostly rich in lipids) and the fact that their numbers frequently increase in old roots, suggest that they are mainly resting and storage organs (Bonfante-Fasolo 1984). These structures may also act as propagules by initiating regrowth of the AM fungus after the metabolism of the roots has ceased (Diop *et al.* 1994).

2.8.9. Life cycle: The life cycle of AM fungi begins when fungal propagules (resting spores, or separated intraradical or extramatrical hyphae) start to germinate (Bago *et al.* 2000). During its limited independent growth period triacylglycerides (TAG) and glycogen, the main C storage compounds of the fungus, are mobilized. This mobilization fuels the development of coenocytic germ tubes and provides C skeletons for anabolism, including the *de novo* synthesis of the chitinous cell wall that surrounds all the fungal structures. Asymbiotic growth is maintained for one or two weeks, during which germ-tube development may reach several centimeters. However, if a symbiosis with a susceptible plant root is not successfully established within this period, AM fungi arrest their growth. Arrest of growth is accompanied by germ-tube septation and nuclear autolysis after which fungal propagules re-enter a state of dormancy and have the ability to re-germinate several times. Growth arrest before complete depletion of C stores may be a strategy to increase the chances of finding an appropriate root to colonize.

If and when the asymbiotically growing AM fungus does contact a host root, a series of signalling events occurs between the partners, which leads to the “acceptance” by the host root of the AM fungus as a symbiont (Smith and Read 1997). Root colonization is accompanied by the development of an extramatrical mycelium that includes characteristic branched absorptive structures (BAS). The external spores develop on some of these BAS completing the fungal life cycle.

Various studies have examined the role of external environmental characteristics that regulate AM formation (Allen 1991). The following postulates were examined in these studies: Phosphate deficiency induces increased membrane leakage, which stimulates fungal colonization; and volatiles secreted by plant roots direct fungal growth. Results from these studies support the idea that when the plant is under stress, it “signals” the fungus to invade and “correct” its deficiency.

2.9. BENEFITS

The formation of AM fungi in root systems has often been shown to be beneficial for host plants, especially under abiotic and biotic stress (Smith and Read 1997). Therefore, when exposed to diverse stress conditions such as limitations in mineral availability, exposure to heavy metals or salt, acidity, drought or attack by pathogens, more than 80% of plants become colonized by AM fungi (Allen 1991). The plant receives a variety of benefits, which may result in increased growth and better adaptation to its environment. These benefits include improved water relations, enhanced nutrient uptake over non-AM controls

and modification in root morphology. In addition, the plant obtains increased protection against environmental stresses, including drought, cold, salinity and pollution. The AM symbiosis also tends to reduce the incidence of root diseases and minimizes the harmful effect of certain pathogenic agents (Allen 1991), while also alleviating metal toxicity, commonly associated with acid soils (Medeiros *et al.* 1995; Clark and Zeto 1996).

An acid soil by definition has relatively high concentrations of hydrogen ions (H^+) and pH *per se* is often not the cause restricting plant growth on acid soils (Clark 1997). The pH often needs to be below 3 before H^+ becomes toxic and soil pH values below 5 are commonly associated with toxicities of aluminum (Al) and manganese (Mn). However, it is known that plant roots can become colonized with AM fungi in soil with high Al levels. Moreover, the acquisition of minerals deficient in acid soils [Ca, Mg and K], other than P and Zn, are enhanced by AM colonization (Clark and Zeto 1996). However, plants grown on acid soils commonly undergo P deficiency as the availability of phosphate is constrained by acidity (pH < 6.5) (Groves 1983). Concurrently, P is the most commonly reported mineral nutrient to be enhanced by AM fungi (Smith and Read 1997).

2.9.1. Phosphorus in soil: Soil P is found in different pools, such as organic and mineral P (Schachtman *et al.* 1998). It is important to emphasize that 20 to 80% of P in soils is found in the organic form, of which phytic acid (inositol hexaphosphate) is usually a major component. The remainder is found in the

inorganic fraction, which may contain up to 170 different mineral forms of this element.

Although the total amount of P in the soil may be high, it is often present in unavailable forms or in forms that are only available beyond the rhizosphere (Allen 1991). Few unfertilized soils release P fast enough to support the high growth rates of certain plant species, e.g., agricultural crops. In many agricultural systems, in which the application of P to the soil is necessary to ensure plant productivity, the recovery of applied P by crop plants in a growing season is relatively low. In soil, more than 80% of the P becomes immobile and unavailable for plant uptake because of adsorption, precipitation or conversion to the organic form.

Compared to more soluble minerals, such as K, that move through the soil via bulk flow and diffusion, P moves mainly by diffusion (Schachtman *et al.* 1998). Since the rate of diffusion of P is slow (10^{-12} to 10^{-15} m^2 s^{-1}), high plant uptake rates thus create a zone around the root that is depleted of P. Thus, the low availability and mobility of P in the bulk soil limits the uptake of this essential macro-nutrient by plants.

2.9.2. Arbuscular mycorrhizae and P-uptake: P is an important plant macro-nutrient, constituting up about 0.2% of a plant's dry weight (Schachtman *et al.* 1998). It is a component of key molecules such as nucleic acids, phospholipids and ATP. Inorganic orthophosphate (Pi) is also involved in controlling key enzyme reactions within metabolic pathways. Consequently, plants cannot grow

without a reliable supply of P, the availability of which is known to frequently limit plant growth in many ecosystems. After N, P is the second most frequently macro-nutrient that limits plant growth. Therefore, plants need to maximize P-uptake mechanisms to counter the limited availability of this macro-nutrient.

Plant root geometry and morphology are important for maximizing P-uptake, because root systems that have higher ratios of surface area to volume will more effectively explore a larger volume of soil (Allen 1991). In this regard, AM fungi are able to absorb P more efficiently than their hosts by increasing the absorptive area of the plant's root system and accessing P sources unavailable to the host roots (Smith and Read 1997). Observations on the early appearance of AM fungi in roots coupled with hypotheses about early terrestrial environments (Allen 1991), led to the proposal that colonization of the land by plants depended in part on the evolution of arbuscular mycorrhizae. The arbuscular mycorrhizae provided essential acids and an uptake mechanism in the form of a mycelium, for the acquisition of P.

The extensive extramatrical mycelium produced by the fungus is highly adapted to an efficient uptake and transport of nutrients, and constitutes a link between the plant roots and the soil environment (Smith and Read 1997). In addition, it extends far beyond the depletion zone into undepleted soil. If it can absorb soluble phosphate ions and transport it to the root, the depletion zone will be effectively bridged and the supply of phosphate ions to the root increased. Several studies have shown that the depletion zone created around plant roots, as a result of plant P- uptake and the immobile nature of Pi, is larger in AM than

in non-AM plants (Bolan 1991). Influx of P into roots colonized by AM fungi can be three to five times higher than in non-AM roots with rates of 10^{-11} mol m^{-1} s^{-1} (Smith and Read 1997). Concurrently, most investigators have found that plants colonized with AM fungi contain a higher concentration of P than do comparable non-AM plants (Krishna and Bagyaraj 1984). Arbuscular mycorrhizal fungi may also be able to acquire P from organic sources that are not available directly to the plant (e.g. phytic acid and nucleic acids) (Jayachandran *et al.* 1992) as these fungi have increased levels of alkaline phosphatase activity (Allen 1991).

2.9.3. Arbuscular mycorrhizal fungi and the solubilization of P: Studies suggest that AM fungi have the capacity to enhance not only the transport of P to a plant from the soil solution, but also to enhance the weathering rates of P from the immobilized, inorganic P pool (Jurinak *et al.* 1986; Knight *et al.* 1989). It was proposed that AM fungi enhance the availability of soil P by weathering the nutrient from the clay matrix and maintaining it in solution by the production of oxalates, which in turn solubilize insoluble complexes of P with Al, Ca and Fe. These soluble oxalate complexes usually form at soil pH values ranging from 5.5 to 7.0. In semi-arid habitats the oxalates may then be degraded by actinomycetes resulting in elevated CO_2 levels, which in turn may enhance P weathering from clay soils (Knight *et al.* 1989).

2.9.4. Transportation and exchange of P: Little is known about the transport of P compounds within arbuscular mycorrhizae or the mechanism of P efflux from

the fungus (Schachtman *et al.* 1998). However, it is believed that Pi in the soil solution is absorbed by the external mycelium via an AM P transporter energized by a P-type H^+-ATPase (Harrison and Van Buuren 1995). The Pi entering the cytoplasm of the AM fungus may be incorporated into phosphorylated primary metabolites, structural molecules and nucleic acids (Viereck e*t al.* 2004). It is assumed that the Pi is then translocated by the external hyphae to the intraradical hyphae in vacuoles within a motile tubular vacuolar system, and condensed into polyphosphate granules, possibly in microbody-like structures. A recent study of AM fungi have confirmed the presence of tubular vacuoles and microtubules (Uetake *et al.* 2002). Once translocated to the symbiotic interface inside the root, the polyphosphate has to be hydrolyzed and the Pi released and transferred to the plant root cells (Viereck e*t al.* 2004). However, there is also evidence in higher plants that phosphocholine, which is effluxed by the fungus to the plant, may be extracellularly degraded resulting in the release of Pi (Schachtman *et al.* 1998). Similar to the uptake process in non-AM roots, the plant would then take up the Pi via a H^+ cotransporter. This transfer is believed to occur at the arbuscular interface, which is in agreement with the recent discovery that plant P transporters are expressed in root cells containing arbuscules (Rausch *et al.* 2001).

2.9.5. Phosphorus demand: In AM roots, demand for P by the plant may regulate the activity of P transporters in the fungus, with efflux from the fungus being the limiting step (Schachtman *et al.* 1998). Therefore, low levels of

colonization seen in plants growing in soil with a high P status may not be the result of direct regulation of the activity of the fungus by soil Pi, but, rather, of the absence of specific signals from the plant, thereby regulating the activity of the fungus.

2.10. ARBUSCULAR MYCORRHIZAL IMPROVED GROWTH

It is well established that AM fungi may increase plant growth, and that the growth improvement is greatest in acidic soils of low fertility (Clark 1997). Consequently, enhanced P nutrition of AM plants growing in phosphate-limited soils usually leads to higher plant growth rates than non-AM plants (Sanders and Tinker 1971; Smith 1982; Bolan 1991; Orcutt and Nilsen 2000). Increased growth accompanied by a higher concentration of a nutrient within the plant provides evidence that AM colonization is thus directly responsible for the increased uptake of otherwise unavailable nutrients (Allsopp and Stock 1993b). However, plant growth may also be depressed as a result of C-costs to the plant exerted by the symbiosis (Cavagnaro *et al.* 2003).

2.11. CARBON-COST OF THE AM SYMBIOSIS

The net result of a symbiosis has mostly been used as a measure of “cost” or “benefit”, with a favorable net result regarded as a benefit and an unfavorable net result as a cost (Koide and Elliott 1989). Carbon is a logical currency to use in such a cost-benefit analysis. Indeed, C may be regarded as the currency plants use to store and transfer energy within the plant body and the currency by which

energy is transferred between the plant and its environment. The C available for the production of standing dry matter is the difference between that which is acquired via gross photosynthesis and that which leaves the plant via respiration, exudation, leaching, death of plant roots, herbivory and symbiotic organisms. Clearly, AM colonization has the potential to affect several of these processes, both above-ground and below-ground.

Interest in the flow of C between the plant and its mycosymbiont has been spurred recently, because it was realized that relatively large amounts of C are involved, making this C flow a fundamental aspect of the symbiosis (Bago *et al.* 2000). Lewis (1973) noted that the fungus is heterotrophic and cannot fix significant amounts of C, but is structurally and physiologically adapted to move inorganic nutrients. In addition, AM fungi may be able to scavenge Pi from the soil solution more effectively than other soil fungi because C, which may be limiting in soil, is provided to the fungus by the plant (Schachtman *et al.* 1998). However, the dependency of the host plant on AM for P acquisition is balanced by the costs of maintaining the association (Mortimer *et al.* 2004). Unfortunately, this cost of the increased P acquisition by the AM root has often been disregarded (Koide and Elliott 1989).

More than 90% of a plant's roots can be colonized by an AM fungus, which can constitute up to 20% of the root dry mass (Mortimer *et al.* 2004). In addition, AM fungi derive most, if not all, of their C from the host plant (Smith and Read 1997). Transfer of organic C below ground due to the sink effect of the biotrophic fungus, is thus a cost incurred by the host as a consequence of colonization

(Kucey and Paul 1982; Snellgrove *et al.* 1982; Koch and Johnson 1984; Jakobsen and Rosendahl 1990). Mortimer *et al.* (2004) demonstrated that the rapid loss of C from colonized roots can also be attributed to the relatively higher growth rate of new fungal structures compared to the growth rate of new root tissue in non-colonized plants. Especially at the seedling stage, AM fungi represent a considerable C drain on a plant (Bethlenfalvay *et al.* 1982). During the phase of rapid development, a greater percentage C will be used for the growth of new fungal structures and only once the symbiosis is functional, will more C be used for nutrient acquisition, such as P (Mortimer *et al.* 2004).

Enhanced P inflow to AM plants (generally regarded as the primary “benefit” of colonization) may be viewed in terms of its effect on increasing photosynthesis (Koide and Elliott 1989). There is evidence that photosynthesis is regulated in great measure by sink strength as plants have been shown to direct 4% to 20% more photo-assimilate to AM root systems than to non-AM roots (Graham 2000). Hence, C loss to the AM fungus could result in a compensatory enhancement of photosynthesis (Koide and Elliott 1989). In the possible case of AM growth depression, reduced growth of AM plants can be viewed as a reduction in the C available for plant growth due to impaired photosynthesis or increased C losses. Furthermore, Baas *et al.* (1989) found that 13% of fungal C was used for increased nutrient uptake and the remaining 87% for fungal respiration. In addition, respiration of AM roots was found to be between 6.6% and 16.5% (depending on fungal species) higher than non-AM roots in cucumber plants, indicating that AM roots have higher metabolic rates than non-AM roots (Pearson

and Jakobsen 1993). Consequently, the increased respiration rate contributes to the sink effect of the fungus (Bago *et al.* 2000). Thus, whether growth of the host plant is increased or decreased is the result of fungal effects on both C assimilation and C expenditure (Koide and Elliott 1989).

Arbuscular mycorrhizae may thus result in an increased allocation of C to the roots and the rhizosphere through increased root growth and respiration and to the bulk soil through hyphal production, respiration and decay (Finlay and Söderström 1992). Therefore, on a global scale the AM symbiosis determines the flow of huge quantities (5 billion tons annually) of C into the soil ecosystem (Bago *et al.* 2000). The cost-benefit analysis and underlying mechanisms are thus of no small ecological and agricultural importance.

The influence of AM colonization (at this stage) may thus be viewed in terms of the effect on host plant C-economy (Koide and Elliott 1989). Moreover, the symbiosis may also be influenced by the species composition of the AM population present, by the hierarchical structure of AM species in the root niche, and by their inherited genetic and functional diversity (Smith and Gianinazzi-Pearson 1988).

2.12. IDENTIFICATION OF AM FUNGI

The diversity of AM fungi in ecosystems has significant ecological consequences, because individual species or isolates vary in their potential to promote plant growth and adaptation to biotic and abiotic factors (Jeffries *et al.* 2003). At the same time, different host plants may differ in their response to

specific fungal strains (Streitwolf-Engel *et al.* 1997; Van der Heijden *et al.* 1998) that may simultaneously colonize a single root segment (Merryweather and Fitter 1998). The correct identification of individual isolates is thus an important prerequisite to the analysis of populations of AM fungi in ecological studies (Jeffries *et al.* 2003).

2.12.1. Identification based on spore morphology: AM fungi are classified in the monophyletic phylum, the Glomeromycota (Schüβler *et al.* 2001). Morphological characters of their large, soilborne spores are the basis for the description of over 150 species of these obligatory biotrophs (Pérez and Schenck 1990). Arbuscular mycorrhizal spore walls, or more accurately, wall complexes, have discrete differences in form, function and histochemical composition, which contribute significantly to species diversity and set glomalean or AM fungi apart from other fungal groups (Morton 1986). Melzer's solution has often been used in taxonomic studies of these fungi because of its ability to selectively stain specific sporocarp structures, including the AM spore walls. However, AM spore morphological characteristics may be difficult to discern and are subject to alterations during spore ontogeny or as a result of parasitism (Clapp *et al.* 1995; Merryweather and Fitter 1998). The hyphal connections of the spores, normally needed for genus determination, may also be lost during isolation procedures (Redecker *et al.* 1997), while the occurrence of spores is not always correlated with root colonization (Clapp *et al.* 1995; Merryweather and Fitter 1998). Therefore, the intraradical structures are better for the identification of the AM

fungi associated with a particular plant than the spores of these fungi scattered in the soil (Merryweather and Fitter 1998).

2.12.2. Identification based on root colonization patterns: In natural communities, root colonization appears to derive more frequently from the mycelial network than from spores (Brundrett and Abbott 1994). Also, since AM fungi are obligate and functionally diverse symbionts, the fungi colonizing the roots of a specific host are likely to have the most relevance to function (Helgason *et al.* 2002). Taking into account that mycorrhizal morphology is influenced by host root structure, the following morphological features are therefore used as taxonomic criteria to identify AM genera: Variations in vesicles (position, abundance, size, shape, wall thickness and wall layers), hyphal branching patterns, the diameter and structure of hyphae (especially near entry points) and the staining intensity of hyphae (dark or faint) (Brundrett *et al.* 1996).

2.12.3. Limitations in morphological characterization: When only hyphal structures are present, it is generally only possible to identify AM fungi to the level of the family or genus (Merryweather and Fitter 1998). In addition, members of some families and several newly described lineages cannot be detected by standard staining methods (Redecker *et al.* 2000). Thus, AM morphological characters do not provide reliable typological criteria for the identification of AM species due to their homogenous morphology (Renker *et al.* 2003). These fundamental problems of identification and classification render

the study of their diversity, particularly in their natural habitat, extremely difficult (Schüßler *et al*. 2001). The development of tools for easy recognition of the mycosymbiont is therefore one of the main challenges in current research on arbuscular mycorrhizae (Bago *et al.* 1998).

2.12.4. Characterization of AM fungi at the molecular level: Molecular techniques that analyze DNA sequences are the most powerful tools employed to study the diversity of AM fungi (Jeffries *et al.* 2003). Consequently, these techniques have been used in combination with morphological or biochemical data to study the diversity of AM fungi in their natural or controlled environments. It was stated that for this purpose, these methods provide greater resolution at the infrageneric level than classical taxonomic methods based on morphology (Helgason *et al.* 1999). In addition, DNA-based methods are not affected by character changes during ontogenesis of organ differentiation (Redecker *et al.* 1997).

One of the most successful approaches has been the analysis of the nuclear ribosomal RNA (rRNA) genes (Bruns *et al.* 1990). The nuclear rRNA gene complex is a tandem repeat unit of one to several thousand copies (Cullings and Vogler 1998). This complex has several domains that evolve at varying rates, and thus have different phylogenetic utilities. The 18S (S, Svedberg sedimentation coefficient) and 28S rRNA genes evolve relatively slowly and are useful in addressing broad phylogenetic hypotheses involving a broad range of organisms. The internal transcribed spacer (ITS) evolves relatively fast and can

be useful in determining interspecific and intraspecific relationships. Within the ITS is a largely ignored coding region, the 5.8S rRNA gene. Similar to the other coding regions, the 5.8S region evolves relatively slowly, but because of its location within the ITS, it is generally used only as an alignment tool during sequence comparison. Recently, however, this region of DNA was shown to contain considerable phylogenetic information, particularly with respect to deep basal branches. Consequently, parts of these genes have already been amplified for AM fungi using conserved, universal sequences as binding sites for the oligonucleotide primers (Simon *et al.* 1992).

Recent studies have used polymerase chain reaction (PCR) techniques in combination with isolate- or group-specific primers to identify the AM taxa within roots (Clapp *et al.* 1995, Helgason *et al.* 1999; Redecker 2000; Wubet *et al.* 2003). Parts of the nuclear rRNA regions of AM fungi have also been amplified and sequenced using universal fungal primers (White *et al.* 1990) and genus-specific primers (Simon *et al.* 1992; Clapp *et al.* 1995). These genus-specific primers were designed to amplify glomalean nuclear rRNA genes directly from colonized roots, while eliminating amplification of plant or non-glomalean fungal DNA (Simon *et al.* 1992; Redecker 2000; Wubet *et al.* 2003). However, most of the specific primers designed and used in these studies on AM diversity in plant roots only amplified representatives of a limited number of glomalean taxa and were therefore of restricted applicability (Clapp *et al.* 1999; Daniell *et al.* 2001; Millner *et al.* 2001). Redecker (2000), however, has reported on the use of a set of specific primers to amplify the ITS regions of six of the seven known clades of

AM fungi from colonized roots. This method had two major advantages above previous methods to study AM molecular diversity and function; the first being that only one AM clade was omitted from this experiment and the second was that the origin of a given sequence can be verified using the 5.8S nuclear rRNA gene dataset (Redecker *et al.* 1999; Wubet *et al.* 2003). It must be noted that this knowledge of the most suitable primer sets may be particularly important in AM biotechnology, especially to identify and select specific AM to be used for the enhancement of crop performance.

2.13. AN OPPORTUNITY TO POSITIVELY MANAGE AM ACTIVITIES

An understanding of the basic principles of *rhizosphere* microbial ecology, including the function and diversity of the microorganisms that reside there, is necessary before soil microbial technologies can be applied (Bolton *et al.* 1992). Mycorrhiza, in particular the arbuscular mycorrhiza, may be pivotal in regulating community and ecosystem functioning (Allen 1991). Their role in the efficient exploitation and cycling of soil mineral resources, their bioprotective role against a number of common soilborne pathogens, as well as their role in the conservation of soil structure, make them instrumental in the survival and fitness of many plant taxa in diverse ecosystems, including many crop species.

The significance of AM fungi to crop plants is generally based on improved survival of individuals following transplantation into an exotic environment, biomass increases, or altered physiology that can be perceived as an improvement, e.g., increased nutrient uptake or increased drought tolerance

(Allen 1991). Especially in intensive plant production systems based on low-input agriculture, when plant growth may be restricted by unfavorable environmental conditions, AM fungi favor the host plant development and can increase yield. In addition, AM fungi can be of special interest in sterilized and artificial substrates, such as that used in horticultural greenhouse and field production. Considering the high economic pressure for practical plant production in recent years, an increase of product quality and yield, or the reduction of costs associated with plant production systems becomes even more important.

The considerable impact of AM fungi on plant growth and health makes management of these fungi essential for sustainable agriculture (Smith and Read 1997). However, the selection of appropriately adapted strains of AM fungi for a particular crop is essential in the development of suitable AM inocula, as fungal strains may differ in their effect on the host plant (Jeffries *et al.* 2003).

Commercial AM inocula have been available to farmers in the United States since the early 1980's (Abbott and Robson 1982). Considering the obligate biotrophic nature of AM fungi, the difficulties in inoculum production have imposed a major limitation to the management of the AM symbiosis in agriculture (St-Arnaud *et al.* 1996). Until now, AM inocula have mostly been produced on greenhouse-grown plants. Usually chopped AM roots, often mixed with the growth media containing hyphae and spores, are used to inoculate new plants. This type of inoculum may contain contaminants, such as plant pathogenic fungi. Other methods such as aeroponic culture and alginate encapsulation of root

fragments have been developed in an attempt to increase the quality and reliability of the inoculum.

To conclude, the significance of this type of symbiotic fungal colonization for plant nutrition, and more generally for plant health, makes it one of the potentially more efficacious biological means of assuring crop profitability and environmental quality with a minimum input of chemicals such as fertilizers or pesticides (Whipps and Lumsden 1994).

2.14. FIGURES

FIG. 2.1. *Agathosma betulina* or round-leaved Buchu. Note the purplish-brown color of the branches, and the white, star-shaped flowers (Van Wyk *et al.* 1997).

(a)

(b)

FIG. 2.2. Examples of *Agathosma betulina* products (Van Wyk *et al.* 1997). *Agathosma betulina* leaves (a) are usually steeped in brandy (Buchu tincture) or (b) infused in other liquids (Buchu infusion). Both of these preparations are employed to treat minor digestive disturbances.

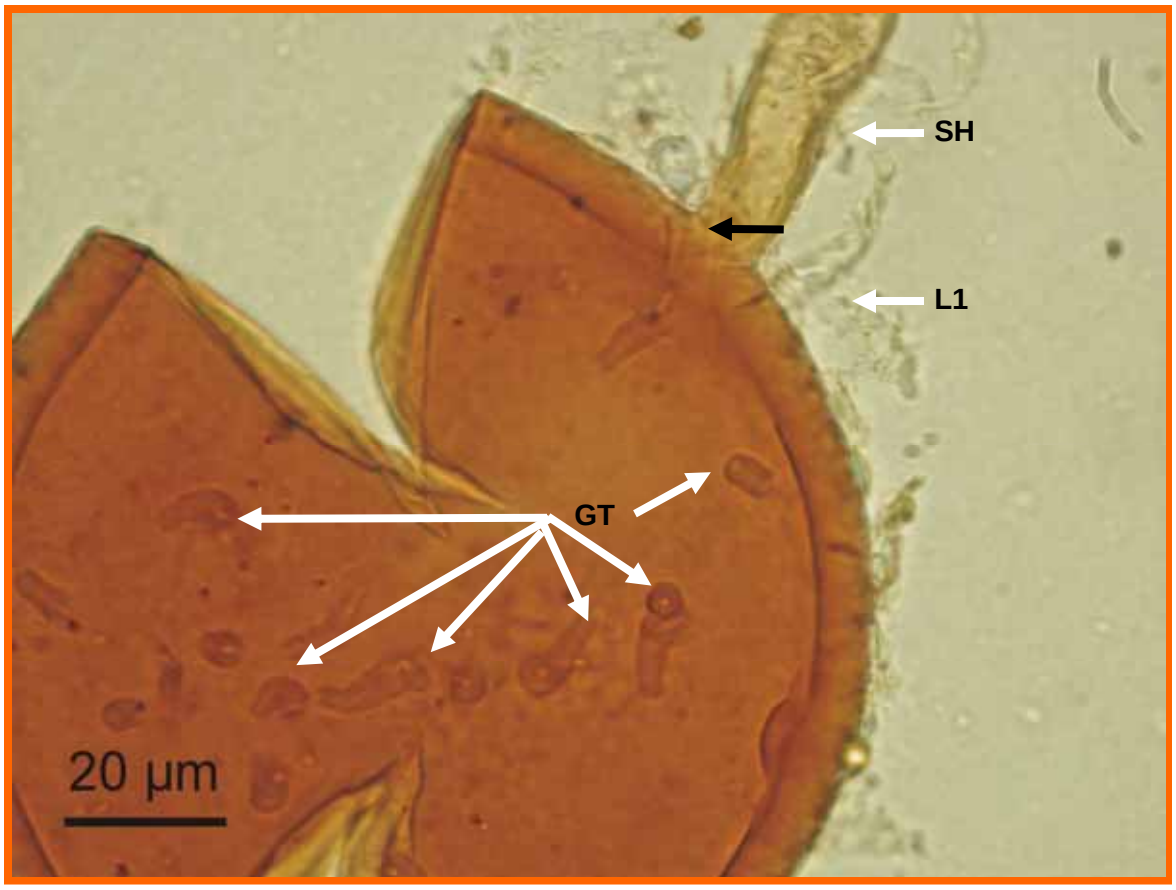

FIG. 2.3. Light micrograph of a single arbuscular mycorrhizal (AM) spore attached to a cylindrical subtending hypha (SH) formed in the rhizosphere soil of pristine stands of *Agathosma betulina.* Note the constrictions formed at the hyphal juncture with the spore wall (black arrow) as well as the continuity between the outer wall layer and the subtending hypha. This mode of spore formation is unique to the genus *Glomus* (Brundrett *et al.* 1996). Several germ tubes (GT) can also be distinguished. In addition, the outermost wall layers are showing signs of sloughing (Layer 1, L1). The AM spore was isolated by means of wet sieving and decanting, followed by sucrose density gradient centrifugation and subsequently stained in Melzer's reagent according to the method described in Brundrett (1994b). Before visual assessment with the use of a compound light microscope, the spore was crushed by applying light pressure on the cover slip covering the stained spore.

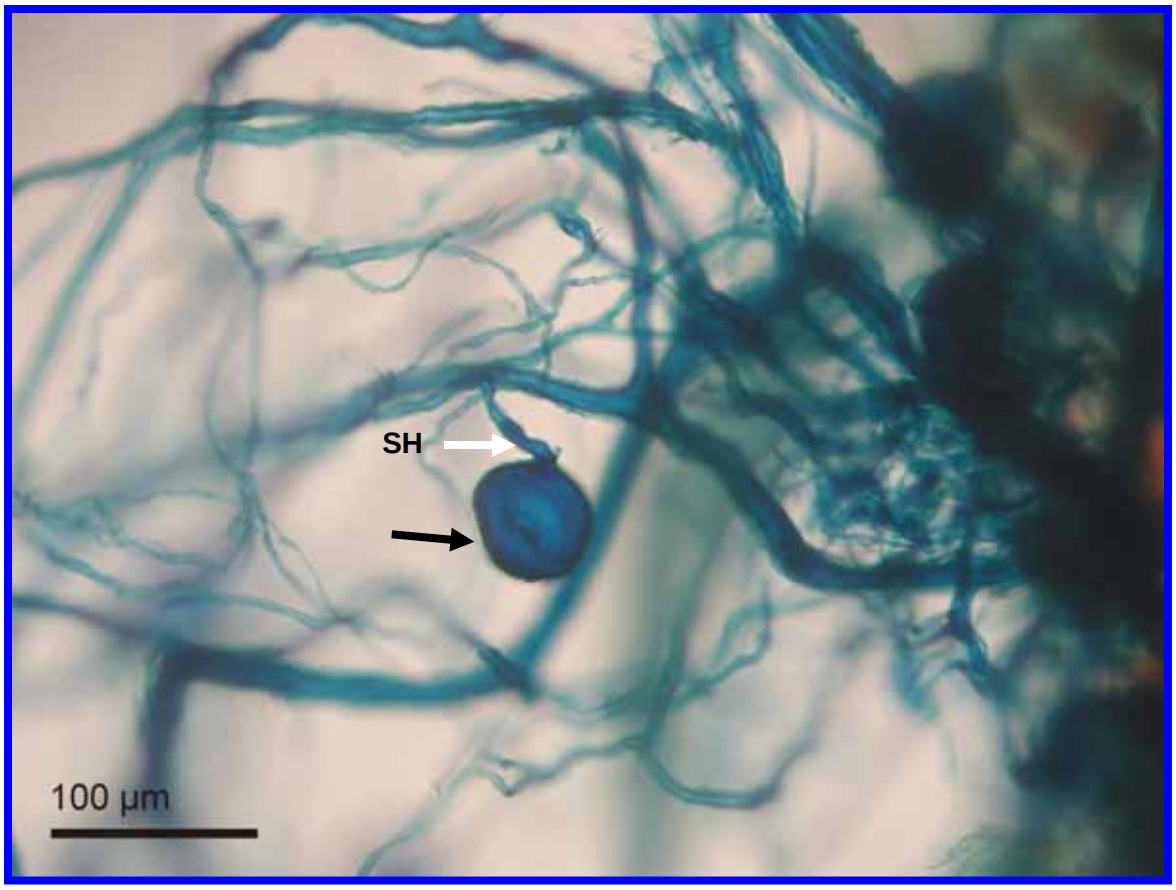

FIG. 2.4. Light micrograph of a smaller arbuscular mycorrhizal spore (black arrow) formed in a hyphal matrix and attached to a subtending hypha (SH). The spore was formed in the rhizosphere of *Agathosma betulina* originating from pristine stands of this plant. The spore was stained with aniline blue according to the method described in Brundrett (1994a).

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CHAPTER 3: Physiological effects of indigenous arbuscular mycorrhizal associations on the sclerophyll *Agathosma betulina* (Berg.) Pillans

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PHYSIOLOGICAL EFFECTS OF INDIGENOUS ARBUSCULAR MYCORRHIZAL ASSOCIATIONS ON THE SCLEROPHYLL *AGATHOSMA BETULINA* (BERG.) PILLANS

3.1. INTRODUCTION

Agathosma betulina (Berg.) Pillans (syn. *Barosma betulina*) is a member of the Rutaceae and popularly known as round-leafed Buchu (Spreeth 1976; Lubbe *et al.* 2003). This slow-growing sclerophyllous, shrubby plant is endemic to the Fynbos vegetation type in the mountainous regions of the south-western Cape, South Africa, which in turn forms part of the Cape Floristic Region (CFR) (Collins *et al.* 1996; Goldblatt and Manning 2000). The plant is cultivated as a source of essential oils, containing amongst others, 8-mercapto-p-menthan-3-one that is used as blackcurrant flavoring in the food, cosmetic and pharmaceutical industries (Collins *et al.* 1996; Lis-Bachin *et al.* 2001; Lubbe *et al.* 2003). In addition, the plant is renowned for its medicinal properties (Van Wyk *et al.* 1997).

Agathosma betulina mainly thrives in podzolic soils derived from quartzites (Spreeth 1976; Kruger 1979). These acidic sandy soils, with a pH ranging from 3.5 to 5.5, are strongly leached, while the nutrients are bound in detritus resulting in oligotrophic soil conditions (Kruger 1979; Stock and Allsopp 1992). The organic carbon content values (C_T) of these soils were found to be *ca.* 1%, while the nitrogen (N) concentration is no higher than 0.06% (Fry 1987). In addition to N deficiency, these soils also contain negligible amounts of available phosphorus (P), with concentrations as low as 3 to 40 μg per gram soil (Kruger 1979).

To overcome these nutrient limitations, CFR plants have, similar to most plants, formed mutualistic symbioses with arbuscular mycorrhizal (AM) or glomalean fungi (Allsopp and Stock 1993a). Surveys of the AM status of plants in the CFR revealed that 62% of indigenous sclerophyllous shrubs form arbuscular mycorrhizae.

Arbuscular mycorrhizal fungi may supply up to 80% of the P and 25% of the N requirements of the host plant (Marschner and Dell 1994). However, it is known that there is a positive correlation between the effectiveness of AM nutrient uptake and the demand of the AM fungus for organic C, obtained from the host (Mortimer *et al.* 2004). Therefore, the net benefit of the AM symbiosis to the plant depends on the benefit derived from uptake of mineral nutrients against the cost of maintaining the mycosymbiont. The latter is calculated in terms of the C supplied by the plant, which is transported below ground due to the sink effect of the AM fungus (Kucey and Paul 1982; Snellgrove *et al.* 1982; Koch and Johnson 1984; Jakobsen and Rosendahl 1990). A factor that contributes to this sink effect is the higher respiration rate and hence metabolic activity of AM colonized roots compared to uncolonized roots (Pearson and Jakobsen 1993). It must be noted that AM species are functionally distinct in, for example, P-uptake and this may in turn impact on the net benefit of the AM association to the plant (Jakobsen *et al.* 1992).

We are interested in the interactions of *A. betulina* with AM fungi present in the native habitat of this plant. This knowledge would provide insight into how sclerophylls rely on AM fungi for survival in their native habitat and would also be

of benefit for large-scale propagation of this plant in nurseries. Consequently, using a mixed inoculum of AM fungi originating from pristine stands of *A. betulina*, we examined the C-costs of AM development and growth nutrition in this sclerophyll. To obtain an indication of the identity of the AM fungi that cause these physiological processes, both morphological and molecular identification techniques were employed to identify AM taxa within the roots of the plant.

3.2. MATERIALS AND METHODS

3.2.1. Sampling site: The sampling site (Fig. 3.1), which comprises an area of *ca.* 500 m^2, is situated on the southern slopes of the Piketberg mountain, at an altitude of 334 to 668 m (32°45'0" S, 18°45'47" E). The site is covered with pristine Mountain Fynbos, an unique collection of indigenous sclerophyllous, evergreen shrubs occurring in mountainous regions, and is adapted to the Mediterranean climate of the south-western Cape region, South Africa (Versfeld *et al.* 1992). The mean annual daily temperature in this cool, temperate, Mediterranean climatic region with a dry summer is *ca.* 17°C (Schulze 1947; Day *et al.* 1979). The mean annual rainfall ranges from 400 to 700 mm and mostly falls in April to August (Day *et al.* 1979). Frost may occur during these months.

3.2.2. Rhizosphere soil sampling and AM spore enumeration: In July 2003 eleven rhizosphere soil samples were randomly taken from young (less than one-year-old) *A. betulina* plants at the sampling site. The samples (*ca.* 400 g each) were collected at depths ranging from 20 to 45 cm, sieved using a 2 mm mesh,

air dried and pooled, resulting in a composite sample consisting of *ca.* 7 kg soil. A 100 g sub-sample of this composite sample was used for chemical content analyses. The bulk of the composite sample was subsequently subjected to cold shock treatment at 4°C for *ca.* 6 weeks to enhance the germination of AM spores dormant at the time of field collection (Vimard *et al.* 1999).

To enumerate and verify the presence of AM spores in the composite soil sample, a 50 g sub-sample was taken prior to cold shock treatment. The AM spores in this sub-sample were isolated using wet sieving and decanting, followed by sucrose density gradient [50% (wt/vol) sucrose] centrifugation (Brundrett 1994c). The soil (50 g) was suspended in 1 l of water for 2 min. with the aid of a magnetic stirrer and allowed to settle for 10 s. The suspension was then decanted onto a nest of sieves (pore sizes of sieves from top to bottom: 2800 μm, 1180 μm, 150 μm and 53 μm) to catch all but the smallest AM spores, yet allow passage of the finest soil particles. Material in the bottom sieve was washed into 2 ml Eppendorf tubes and centrifuged at 1000 × *g* for 5 min. at 4°C in a micro-centrifuge (Biofuge 13, Heraeus Instruments, Inc., South Plainfield, NJ, USA). The supernatants were discarded and the pellets re-suspended in 40% (wt/vol) sucrose. These samples were then centrifuged at 1000 × *g* for 1 min. at 4°C in a micro-centrifuge. Spores and other organisms such as nematodes and mites remained in the supernatant, which was poured onto a sieve, rinsed with water and washed into a Petri dish for initial observation (Millner 1987). Spore suspensions were subsequently pipetted onto moist cellulosic membrane filters (0.22 μm pore size; Micronsep, Massachusetts, USA)

for enumeration purposes (Brundrett 1994c). Enumeration of AM spores was done using a Nikon SMZ 10A dissecting microscope [Innovative Met Products (Pty) Ltd., Boksburg, S.A.] (Magnification, × 73).

3.2.3. Chemical analyses of soil: Available P was analyzed according to the methods proposed by Olsen and Sommers (1982), total C content was analyzed following Nelson and Sommers (1982), and total N concentration was determined using a LECO FP528 Nitrogen Analyzer [Leco Africa (Pty) Ltd., S.A.; Colombo and Giazzi 1982] with Spectrascan standards (Drobak, Norway). In addition, pH (KCl) was determined according to methods proposed by McLean (1982).

3.2.4. Sampling of *A. betulina* roots from Fynbos: By carefully tracing the root system from the main stems to young, primary roots, root samples of *A. betulina* plants (*ca.* 0.4 m) from which the rhizosphere soil samples originated, were collected 48 h before initiation of potting experiments. To verify AM colonization of these roots, a sub-sample of the roots originating from each plant was taken, carefully rinsed in distilled water, fixed in 50% (vol/vol) ethanol (Brundrett 1994a) and the percentage root length colonized by AM fungi assessed as explained under the heading: “3.2.7. Assessment of percentage root length colonized by AM fungi”. The remainder of the root samples (*ca.* 8 g wet weight) was pooled and macerated with scissors resulting in root lengths of *ca.* 1 mm.

3.2.5. Staining of intraradical AM structures: Each root sample fixed in 50% (vol/vol) ethanol was carefully rinsed in distilled water and patted dry with paper towels. The roots were then cleared in 10% (wt/vol) KOH at 121°C for 7 min. (Brundrett 1994a). To rid the cleared roots of excess KOH, it were rinsed four times with tap water and subsequently acidified for 12 h in 2% (vol/vol) HCl. To stain the AM intraradical structures, acidified roots were submerged in 0.05% (wt/vol) aniline blue (Sigma, Sigma Chemical Co., S.A.; Color Index No. 42755) in lactic acid/glycerol/water (14:1:1, vol/vol/vol) at 121°C for 3 min. To stain arbuscules, the roots were submerged in 1% (wt/vol) chlorazol black E (Sigma; Color Index No. 30235) in 80% (vol/vol) lactic acid/glycerol/water (14:1:1, vol/vol/vol) at 121°C for 3 min. In each case, the staining solution was subsequently decanted and the roots de-stained for 14 h at 22°C in an acidified 50% (vol/vol) glycerol solution.

3.2.6. Slide preparation: The stained roots of each sample was cut into 10 mm lengths, which were placed on four slides so that each slide contained 25 parallel root segments (Brundrett 1994b). Using polyvinyl alcohol-lactic acid-glycerine (PVLG) as mountant, the root segments were covered with a cover slip (Fig. 3.2). The composition of the PVLG was as follows; polyvinyl alcohol, 8.33 g; distilled water, 50 ml; lactic acid, 50 ml and glycerine, 5 ml.

3.2.7. Assessment of percentage root length colonized by AM fungi: The percentage root length colonized by AM fungi was determined using the

mathematical equations as described by Brundrett 1994b: *AC* = 100 *(q+s/G)*; *VC* = 100 *(r+s/G)*; *HC* = 100 *(G-p/G)*, where *AC* is the percentage root length colonized by arbuscules (arbuscular colonization), *VC* is the percentage root length colonized by vesicles (vesicular colonization) and *HC* is the percentage root length colonized by hyphae (total or hyphal colonization). The variable *q* represents the number of intersections (hairline graticule inserted into eyepiece acted as the line of intersection with each root segment) containing arbuscules, *s* is the number of intersections containing arbuscules and vesicles, *r* is the number of intersections containing vesicles, *p* is the number of root intersections devoid of AM structures, while *G* represents the total amount of intersections inspected (100). All enumeration was conducted in triplicate. Concurrently, AM structural diversity was studied using a compound light microscope (Magnification, × 200).

3.2.8. Arbuscular mycorrhizal inoculum preparation: To counteract a possible low AM inoculum potential, the composite soil sample (*ca.* 7 kg) containing AM propagules, including spores, was thoroughly and gently mixed with macerated *A. betulina* root fragments (*ca.* 8 g) colonized with AM fungi prior to initiation of potting experiments. Arbuscular mycorrhizal root pieces may contain vesicles, which have a higher potential to establish an AM association with a host than other AM propagules, i.e. spores and hyphae (Diop *et al.* 1994).

3.2.9. Seed germination: Seeds of *A. betulina* were obtained from commercial growers and stored at room temperature. The seeds were surface sterilized with 0.05% (wt/vol) sodium hypochlorite and two subsequent washes in distilled water. Prior to germination, seeds were incubated for 20 min. at 80°C (Blommaert 1972). Seeds were selected for uniformity and allowed to germinate for three weeks in glass Petri dishes on sterile, damp filter paper incubated at 22°C, alternating with 8 to 10 h at 6°C for a total period of 14 to 16 h.

3.2.10. Plant growth and AM inoculation: During August 2003, 24 pot cultures were prepared by planting germinated seeds (four per pot) in 13-cm-diameter free-draining plastic pots, each containing 650 ml potting mixture. The potting mixture consisted of the composite soil sample subjected to cold shock treatment along with colonized *A. betulina* root fragments (the AM inoculum) and autoclaved inert silica sand (grain size 0.5 mm) mixed in a 1:3 ratio to facilitate drainage. The potting mixtures had a pH of *ca.* 5, similar to field collected soil samples. Control cultures were also included in the experimentation. These cultures were prepared in a similar manner, except that the soil was gamma-irradiated [minimum absorbed dose, 25 kGy (kGy = 0.1 megarad) per kg soil] in order to eliminate indigenous populations of AM fungi. To reduce potential phytotoxic effects, they were left to equilibrate at room temperature for one week before planting (Bryla and Duniway 1997). Reconstitution of the associated soil microflora, without AM propagules, in the controls was done during planting by addition of 100 ml soil filtrate (Schubert and Hayman 1986). The latter was

prepared by suspending 50 g rhizosphere soil in 1 l of distilled water and filtering it using a sieve (pore diameter, 53 μm). Pots were arranged in a random design on benches in a well-ventilated glasshouse with 14 h photo-periods (photosynthetic photon flux density 1000 to 1100 μmol m^{-2} s^{-1}). The average day/night temperatures and relative humidities (RH) were 23/15°C and 50/80% respectively. The plants were wetted to field capacity with half-strength Long Ashton nutrient solution at pH 6 (Hewitt and Smith 1975). This was done because small amounts of P applied with AM inoculation may benefit the plant more than either AM inoculation or P application alone (Abbott and Robson 1977). In addition, as dilution of the soil implemented in the potting experiments with silica sand may have decreased P levels, the concentration of P present may have been so low that AM fungi can not develop extensively.

3.2.11. Harvesting and nutrient analyses: Harvesting took place after 77, 157 and 224 days of growth. Plants were randomly harvested and separated from the soil using a gentle spray of tap water and patted dry with paper towels. Upon harvesting, the plants were separated into shoots and roots and the fresh weight of each was determined. The harvested material was then dried at 80°C in an oven for one week, after which the dry weight (DW) was measured. The dried material was milled using a 0.5 mm mesh (Arthur H. Thomas, Philadelphia, Pa., USA). Milled samples were analyzed for their respective C, N and P concentrations. Total C content was analyzed according to methods proposed by Allison (1965), total N concentration was determined using a LECO FP528

Nitrogen Analyzer with Spectrascan standards, and total P was determined using a Vista inductively coupled plasma-atomic emission spectrometer (ICP-AES) [Varian (Pty) Ltd., Aus.; Munter and Grande 1981].

It must be noted that random sub-samples of the harvested roots were taken prior to drying. These sub-samples were obtained by cutting the sampled root material into 1 cm strips and then randomly selecting samples and fixing them in 50% (vol/vol) ethanol for storage. Percentage root length colonized by AM fungi in these fixed root samples was calculated as was described previously under the heading: “3.2.7. Assessment of percentage root length colonized by AM fungi”.

3.2.12. Calculations: Relative growth rate (RGR; mg g^{-1} day^{-1}): For each growth period, the RGR was calculated from the change in DW (mg) of plants relative to their existing or initial weights (g).

Phosphorus-uptake (mol P mg^{-1} day^{-1}): Phosphorus-uptake for each growth period was calculated from the changes in P concentration (mol P g^{-1}) divided by the RGR (mg g^{-1} day^{-1}), to represent the P inflow that was required during a particular growth rate.

Phosphorus utilization cost (C:P) was expressed using the equation proposed by Koide and Elliot (1989) to calculate the quantity of C accumulated in the plant divided by the quantity of P accumulated in the plant for a given period of time: $\Delta C^{r}/\Delta P^{r}$, where ΔC^{r} is the total C accumulated in the plant over a given time period and ΔP^{r} is the total P accumulated in the plant over the same time period.

It should be noted that a low efficiency value indicates that less C is required for the given amount of P utilized by the plant.

Plant construction cost (mmol C gDW^{-1}) was calculated from the tissue construction cost, modified from the equation used by Peng *et al.* (1993): $Cw = \{C + [(kN/14) \times (180/24)]\} (1/0.89)(6{,}000/180)$, where *Cw* is the construction cost of the tissue (mmol C gDW^{-1}), *C* is the carbon concentration of the tissue (mmol C g^{-1}), *k* is the reduction state of the N substrate in the nutrient solution (NH_4 was used, therefore *k* is +3) and *N* is the organic nitrogen content of the tissue (g gDW^{-1}) (Williams *et al.* 1987). The constant (1/0.89) represents the fraction of the construction cost that provides reductant that is not incorporated into biomass (Williams *et al.* 1987; Peng *et al.* 1993) and (6,000/180) converts units of g glucose gDW^{-1} to mmol C gDW^{-1}.

Growth respiration (mmol CO_2 gDW^{-1}) representing the C respired for the biosynthesis of new tissue was calculated as proposed by Peng *et al.* (1993): $Rg(w) = Rg(t)/root\ gr$, where *Rg(w)* represents growth respiration based on DW, *root gr* is the root growth rate (gDW day^{-1}) and *Rg(t)* is the daily growth respiration (μmol CO_2 day^{-1}): $Rg(t) = C_t - \Delta W_c$, where C_t (μmol CO_2 day^{-1}) is the C required for daily construction of new tissue. The variable, C_t was calculated by multiplying the root growth rate by tissue construction cost. The change in root C content, ΔW_c (μmol day^{-1}) was calculated by multiplying the root C content and the root growth rate.

3.2.13. Statistical analyses: All the percentage data were arcsine transformed (Zar 1999) prior to statistical analysis. The differences in percentage AM colonization between harvests (n = 4 for each treatment) were separated using a post hoc Student Newman Kuels (SNK) multiple comparison test ($P \leq 0.05$) implemented in the software program Statgraphics, version 7 (http://www.statlets.com/stagraphics.htm; Statgraphics Corp., USA). For each harvest, the difference between the means of AM and non-AM plants was separated using a Student's t-test implemented in the software program Statistica, version 6.0. (www.statsoft.com; StatSoft, Inc., Tulsa, Okla., USA) for independent samples by groups ($P \leq 0.05$).

3.2.14. Genomic DNA extraction and purification: After 224 days of growth, genomic DNA was extracted from pooled *A. betulina* pot culture root samples. The roots were gently washed under an aerated stream of distilled water in order to remove adhering soil particles and debris, and patted dry with paper towels. Fine roots were chosen randomly for DNA extraction according to an adaptation of the method described by Hoffman and Winston (1987). Freshly harvested roots (*ca.* 1 g) were frozen in liquid N and ground into a fine powder using a pre-cooled (-20°C) mortar and pestle. Subsequently, 0.3 g of the powder was transferred to a 2 ml Eppendorf tube, which was kept on ice, and re-suspended in 400 μl STE-buffer [Tris-HCl 1 M, pH 7.2; NaCl 5 M; EDTA (ethylene-diamine-tetra-acetic acid) 0.5 M, pH 8; distilled H_2O]. Tris-saturated phenol (400 μl, pH 7) was then added to the suspension and the mixture was briefly vortexed. After

the addition of 400 μl chloroform/iso-amyl-alcohol (24:1, vol/vol) the mixture was centrifuged at 13.7 × *g* for 10 min. in a micro-centrifuge at 4°C. The aqueous supernatant was transferred to a clean 2 ml Eppendorf tube. Proteins were denatured by the addition of 1 volume phenol/chloroform/iso-amyl-alcohol (25:24:1, vol/vol/vol). The mixture was vortexed and centrifuged at 13.7 × *g* for 10 min. in a micro-centrifuge at 4°C. The aqueous phase was transferred to a clean 2 ml Eppendorf tube, 40 μl sodium acetate (3 M, pH 5.2) and 1.5 ml 100% (vol/vol) ethanol added, and the DNA precipitated for 14 h at -20°C. The DNA was pelleted by centrifugation at 13.7 × *g* for 10 min. in a micro-centrifuge at 4°C. The supernatant was discarded and the pellet washed with 70% (vol/vol) ethanol. Excess ethanol was allowed to evaporate for 5 min. at 22°C. The brown-colored pellet was subsequently dissolved in 50 μl milliQ water and stored at -20°C.

To assess the degree of purity and degradation, samples containing the isolated DNA were examined by electrophoresis on 0.8% (wt/vol) agarose gels containing ethidium bromide (1 μg/ml) at 90 mV for 1 h. The DNA fragments from successful reactions were purified by means of Microspin S-300 HR columns (Separations Scientific, Amersham Pharmacia Biotech, Inc., S.A.) following the manufacturer's protocol (Separations instruction manual, Separations Scientific) and diluted 10-fold in milliQ water, where necessary. An *EcoR*I/*Hind*III digest of lambda DNA was used as size marker.

3.2.15. Amplification of nuclear ribosomal RNA (rRNA) genes: Fragments of the nuclear rRNA gene complex, including a section of the small subunit (SSU)

or 18S (S, Svedberg sedimentation coefficient) rRNA gene, the internal transcribed spacer region (ITS1, *ca.* 113 to 121 bp), 5.8S rRNA gene (*ca.* 161 bp) and ITS2 region (*ca.* 222 to 230 bp) (Lloyd-Macgilp *et al.* 1996) were amplified using Takara EX *Taq* DNA polymerase (Takara BioCo., Shiga, Jp.). Amplification of the above-mentioned nuclear rRNA genes was performed in a GeneAmp PCR system 2400 (Perkin-Elmer Corp., Norwalk, Conn., USA) in a total volume of 25 μl containing 0.8 μl Takara EX *Taq* DNA polymerase (5U/μl), 0.08 mM dNTP (Takara BioCo.), 20 pmol of each primer [Inqaba Biotechnical Industries (Pty) Ltd., S.A.], 2.4 mM $MgCl_2$, 80 mM KCl, 16 mM Tris-HCl (pH 8.3), 5 μl formamide [5% (vol/vol)], and 0.9 μl genomic DNA (10-fold diluted in milliQ water). A similar procedure was followed for control reactions, where an equal volume of milliQ water was added to the reaction mixture instead of the template DNA. The PCR program comprised the following cycles: An initial denaturation step for 5 min. at 94°C; 5 cycles (45s at 94°C; 30s at 61°C; 2 min. at 72°C), and 25 cycles (45s at 94°C; 30s at 60°C; 2 min. at 72°C). A final elongation of 7 min. at 72°C followed the last cycle.

An universal fungal primer pair was used in the first reaction of the nested PCR. These primers were NS5 (5'-AACTTAAAGGAATTGACGGAAG-3'; White *et al.* 1990) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.* 1990). After the first PCR, the reaction mixtures were diluted 1000-fold and used as templates in the next amplification step. The second reaction of the nested PCR was performed with various AM specific primers in combination with universal fungal primers (ITS1 and ITS4). Primer pairs for the second amplification

reaction consisted of the universal fungal primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; White *et al.* 1990) in combination with the reverse AM specific primer GIGA5.8R (5'-CACATGCTTGAGGGTCAGT-3'; Redecker 2000) and the universal fungal primer ITS4 in combination with each of the following forward AM specific primers: ACAU1660 (5'-TGAGACTCTCGGATCGGG-3'; Redecker 2000), ARCH1311 (5'-TGCTAAATAGCTAGGCTGY-3'; Redecker 2000), GLOM1310 (5'-AGCTAGGCTTAACATTGTTA-3'; Redecker 2000) and LETC1670 (5'-ATCGGCGATCGGTGAGT-3'; Redecker 2000). The relative positions of the annealing sites of these primers are depicted in Fig. 3.3. These AM specific primers are known to amplify selected AM taxa as depicted in Table 3.1.

In order to estimate the quantity of PCR product, the amplification products were separated electrophoretically on 1% (wt/vol) agarose gels, containing ethidium bromide (1 μg/ml) at 90 mV for 1h. An *EcoR*I/*Hind*III digest of lambda DNA was used as size marker. Prior to cloning, bands obtained during the second reaction of nested PCR were visualized by UV transillumination and excised using a sterile scalpel blade. Excised DNA fragments were immediately purified by means of a High Pure PCR Product Purification Kit [Roche Molecular Biochemicals (Pty) Ltd., S.A.) following the manufacturer's protocol (Roche instruction manual, vol. 2, Sept. 1999; Roche Molecular Biochemicals).

3.2.16. Cloning and sequencing: As most PCR products amplified with Takara EX *Taq* (Takara BioCo.) have one adenine added at the 3'-termini, the obtained

PCR products can be directly cloned into a T/U-vector (Lloyd-Macgilp *et al.* 1996). The purified PCR products were cloned into the pDRIVE Cloning Vector by means of the Qiagen PCR Cloning^{plus} Kit [Qiagen, Southern Cross Biotechnology (Pty) Ltd., S.A.] and transformed into EZ Chemically Competent *Escherichia coli* (XL1-blue) cells (Qiagen) following the manufacturer's protocol (PCR cloning handbook, Qiagen). Putative positive transformants were selected and recombinant plasmid DNA isolated by means of QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol (Qiaprep miniprep handbook, Qiagen). To verify the presence of the expected PCR fragment, plasmid DNA was subsequently digested with *EcoR*I (Takara BioCo.) following the manufacturer's instructions (Takara manual, Takara BioCo.).

Sequencing was done by the dideoxy chain termination method, using an ABI Prism model 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif., USA) and T7/SP6 as sequencing primers. The sequences were analyzed and edited using the PC-based software program DNAMAN, version 4.13. (http://www.lynnon.com/pc/framepc.html; Lynnon Biosoft Corp., USA). Homologues to the isolated sequences were identified using the software program BLASTN (nucleotide-nucleotide BLAST), version 2.2.9. [freeware: http://www.ncbi.nlm.nih.gov/BLAST; National Center for Biotechnology Information (NCBI), USA; Altschul *et al.* 1997].

3.2.17. Nucleotide sequence accession numbers: Sequences obtained from AM colonized roots of *A. betulina* were deposited in the GenBank data library

under the following accession numbers: AY856454 (Sequence 1; *Glomus* sp. isolate 1), AY856455 (Sequence 2; *Glomus* sp. isolate 2), AY856456 (Sequence 3; *Glomus* sp. isolate 3) and AY856457 (Sequence 4; *Acaulospora* sp. isolate 4).

3.2.18. Phylogenetic analyses: Alignment of full length 5.8S nuclear rRNA gene sequences obtained during the study, as well as from all families within the phylum Glomeromycota, obtained from GenBank, was carried out using the software program BioEdit, version 7.0.1. (freeware: http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall 1999) with final editing being performed manually. *Nuclearia simplex* (Patterson 1984) was included as an outgroup. The aligned 5.8S nuclear rRNA gene dataset was transferred to the software program PAUP (phylogenetic analysis using parsimony), version 4.0. beta 10 for a Macintosh G5 (http://paup.csit.fsu.edu/; Sinauer Associates, Inc., Sunderland, Mass., USA; Swofford 2000) for phylogenetic analysis. Phylogenetic trees were inferred using the parsimony criterion implemented in PAUP, with all characters equally weighted. Nodal support was assessed using 1000 bootstrap replicates (Felsenstein 1985) with simple taxon addition and tree-bisection-reconnection branch swapping.

As inclusion or exclusion of a few characters can greatly affect the bootstrap proportions of maximum-parsimony trees derived from limited datasets (Koper *et al.* 2004), we also conducted a model-based search by subjecting the aligned 5.8S nuclear rRNA gene dataset to a Bayesian analysis, using Monte Carlo Markov Chains (MCMC) as implemented in the software program MrBayes,

version 3.0. beta 4 (freeware: http://morphbank.cbc.uu.sc/mrbayes/; Huelsenbeck and Ronquist 2001). Monte Carlo Markov Chains of trees are constructed such that, after stationary is reached, it can be used to approximate the posterior probability that groups of taxa are monophyletic, given the DNA alignment, i.e. the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species (Wubet *et al.* 2003).

In developing a Bayesian phylogenetic hypothesis, a model of evolution (DNA substitution) needs to be adopted (Baldi and Brunak 2001). Consequently, the software program Modeltest, version 3.6. (freeware: http://darwin.uvigo.es/software/modeltest.html; Posada and Crandall 1998) was used to select the most suitable model of evolution. Modeltest selected the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al.* 1985) with gamma distributed rate variation among sites (Yang 1996) as the optimal model for the aligned 5.8S nuclear rRNA gene dataset. This model was thus implemented in MrBayes.

Four incrementally heated simultaneous MCMC were run for 1 000 000 generations using the parameter for DNA site substitution (“nst = 2” for the HKY-model of evolution), a random starting tree and default parameters settings. This was done to allow successful swapping between MCMC, and to build a sufficient number of reliable trees after the log likelihood values of the MCMC have converged on a stable value. Trees were sampled every 50 generations, resulting in a total of 20 000 trees sampled. The “sump” command was used to

assess the number of trees sampled before the log likelihood values of the MCMC reached stationary. By ignoring the trees generated before the search converged on stable log likelihood values of the MCMC (removed by the “burnin” command), MrBayes summarized the results in a post-run analysis concerning tree topology. The remaining trees were used to generate a 50% majority rule Bayesian consensus tree topology (also including compatible groups of lower frequencies) and Bayesian posterior probability estimates (i.e., the percentage of the time a particular clade occurred), which were recorded at the nodes. To test the reproducibility of the results, computations were repeated three times using the parameter for DNA site substitution, a random starting tree and default parameters settings.

Phylogenetic trees were viewed with the software program TreeView, version 1.6.6. (freeware: http://taxonomy.zoology.gla.ac.uk/rod/treeview.html; Page 1996) and edited with Microsoft Paint, Windows 95.

3.3. RESULTS

3.3.1. Chemical analyses of soil: The composite soil sample collected from the rhizosphere of *A. betulina* plants at the sampling site was an acidic (pH 4.54 ± 0.18) sandy loam, with low available P levels (5.12 ± 0.46 mg/kg). In addition, it contained low levels of N (0.15 ± 0.01%) and C (2.70± 0.19%).

3.3.2. Arbuscular mycorrhizal inocula: An AM spore concentration of 250 ± 42 AM spores per 50 g dry, sieved soil was observed in the composite sample

taken during the wet, rainy season of July 2003. Spore morphology, as studied using a dissecting microscope (magnification, × 73), indicated that the majority of AM spores in the composite sample were healthy and unparasitized (Fig. 3.4). Arbuscular mycorrhizal spores isolated varied in color from olive yellow to dark red and had shiny surfaces (Fig. 3.4). The percentage root length colonized by AM fungi (hyphal colonization) of the *A. betulina* root segments in the inoculum was low (34 ± 2.89%).

3.3.3. Arbuscular mycorrhizal colonization of pot grown *A. betulina*: All stages of arbuscular mycorrhiza (appressoria, intraradical hyphae, arbuscules, and vesicles) were present at 77 days after inoculation, whereas non-AM plants remained non-AM for the duration of the experiment. The growth period 0 to 77 days showed the highest rate of AM colonization (Fig. 3.5). Following this period, AM colonization leveled off (days 77 to 157) and then declined towards the end of the experiment, after 224 days of growth (Fig. 3.5). Changes in the percentage arbuscular colonization followed the same trend as the percentage hyphal colonization, while the percentage vesicular colonization showed a constant increase over the measured growth periods (Fig. 3.5). It is also interesting to note that the percentage hyphal colonization in 224 day old plants (Fig. 3.5) and root segments obtained from *A. betulina* plants collected in the wild at the sampling site, were similar (see: “3.3.2. Arbuscular mycorrhizal inocula”).

3.3.4. Growth and nutrition: Between 0 and 77 days of cultivation the RGR of AM plants was lower than non-AM plants (Fig. 3.6a), which resulted in smaller biomasses of AM plants at the 77 days harvest (data not shown). From days 77 to 224, the RGR of AM plants increased to similar rates as the non-AM plants (Fig. 3.6b, c) and resulted in similar biomasses (data not shown). At day 77, P concentration reached a maximum in AM plants compared to non-AM plants (data not shown). Plant P-uptake rate was higher in the AM plants up to day 77 and then declined to equal levels with non-AM plants at days 157 to 224 (Fig. 3.7a).

3.3.5. Respiratory C-costs: The P utilization cost, which expresses the efficiency of C used for P acquisition, was lower in the AM plants than the non-AM plants at days 0 to 77 and days 77 to 157 (Fig. 3.7b). Subsequent to these growth periods, the P utilization cost for AM plants were significantly ($P \leq 0.05$) higher, compared to non-AM plants at days 157 to 224 (Fig. 3.7b). The growth respiration of AM roots was significantly ($P \leq 0.05$) higher than the non-AM control roots at day 77 (Fig. 3.7c). However, this higher growth respiration reached a level equal to non-AM roots at days 157 to 224 (Fig. 3.7c).

3.3.6. Morphological features of AM structures within sampled roots: Clearing performed in the autoclave was sufficient to remove phenolic compounds, pigments and cell contents without damaging or disintegrating the cortex cells. Initial AM colonization of *A. betulina* roots occurred via an

appressorium (Fig. 3.8). The staining technique employed in the study yielded appropriate contrast between the root cortex and the AM structures, especially the arbuscules (Fig. 3.9). Intracellular arbuscules characteristic of the genus *Glomus* (Fig. 3.9), arising from extensions of linear intracellular hyphae, usually stained with a wide range of intensity, from almost invisible to dark. However, the arbuscular network was often hard to find in the older roots obtained from the sampling site, as well as in 157 to 224 day old sampled roots obtained from pot-cultured plants, as it was mostly replaced by arbuscular clumps (Fig. 3.9) or a hyphal network with or without numerous vesicles and/or intraradical spores (Fig. 3.10). Darkly stained, oval shaped, and thin-walled vesicles started to form immediately after AM root entry and the formation of these structures continued to occur until late colonization (Fig. 3.5). The vesicles usually formed between root cortex cells and were attached to intraradical hyphae. Two types of intraradical hyphae were observed, namely the intercellular *Arum*-type and the intracellular *Paris*-type (Smith and Smith 1997). These two types were, respectively, characterized by relatively parallel darkly stained intercellular colonization hyphae, proliferating along the longitudinal axis of the root (Fig. 3.11) and the formation of intracellular coils in the cortical tissue near entry points (Fig. 3.12). The former was cross-connected with branched hyphae (“H” connections) at varying angles (Fig. 3.11). Root colonization patterns and diagnostic structural features of the AM fungi present were similar in both pot grown plants and plants obtained from the sampling site, and in both cases were

characteristic of *Glomus clarum* (Nicolson and Schenck 1979) and *Glomus intraradices* (Schenck and Smith 1982).

3.3.7. Genomic DNA extraction and purification: At harvest, after 224 days of growth, hyphal colonization of the pot grown *A. betulina* was *ca.* 20% (Fig. 3.5). The method employed to extract the DNA did not require any lengthy procedure, yet it provided sufficiently abundant and pure DNA templates of the appropriate size to ensure successful PCR.

3.3.8. Amplification of nuclear rRNA genes: Preliminary experiments indicated that optimal amplification results were obtained when roots were processed for PCR immediately after harvesting. Storage of roots in the refrigerator, or processed DNA samples in the freezer (-20°C) for more than 2 weeks, diminished or eliminated amplification of the initial PCR product. The optimal cycling conditions and concentration of the components used in the PCR were determined after testing a number of different conditions. Template DNA concentration was one of the decisive parameters. Preliminary experiments also indicated that using *Taq* polymerase and standard PCR conditions yielded negative results, whereas positive results were obtained with Takara EX *Taq* polymerase. However, nonspecific products were routinely amplified during the first reaction of nested PCR incorporating the fungal specific primers, NS5 and ITS4. The presence of these DNA products indicated that, under the conditions used, the primer pair was not taxon specific and would amplify non-AM DNA.

Control experiments where no DNA template was added to the reaction mix usually gave no amplification product. Thus, to dilute high concentrations of multiple, non-specific products obtained during the first amplification reaction, the products of this reaction were diluted 1000-fold before it was used as template for the second amplification reaction of nested PCR. In addition, formamide was included in order to increase specificity (McPherson and Mølller 2000) of the second reaction of nested PCR, conducted with AM specific primers in combination with fungal specific primers. Amplification products (*ca.* 1 kb bands) were consistently amplified incorporating the nested primer pairs GLOM1310/ITS4 and ACAU1660/ITS4, whereas negative results were obtained in other nested PCR reactions.

3.3.9. Cloning and sequencing: PCR products obtained from the second reaction of nested PCR were cloned as previously sequenced excised products yielded uninterpretable results. The four sequences obtained from AM colonized *A. betulina* roots (see: “3.2.17. Nucleotide sequence accession numbers”) varied in length from 780 bp to 966 bp and a BLASTN search indicated that the sequences were of glomalean origin.

3.3.10. Phylogenetic analyses: Phylogenetic analyses were done to verify the glomalean origin of the 5.8S nuclear rRNA gene sequences obtained during the study, to show the systematic position of these new sequences, as well as to illustrate the overall molecular diversity of AM fungi associated with *A. betulina*.

We restricted the analyses to full length 5.8S nuclear rRNA gene sequences representing seven AM genera (i.e., *Acaulospora*, *Archaeospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Paraglomus*, and *Scutellospora*), the representative sequences obtained during this study, and their corresponding closest matches from GenBank.

Characteristics of the full length 5.8S nuclear rRNA gene region of the analyzed taxa are summarized in Table 3.2. Parsimony analysis produced 14 equally parsimonious trees with a consistency index of 0.699 and a retention index of 0.814 for the strict consensus of these 14 trees, which indicate reasonably low levels of homoplasy (Swofford and Olsen 1990). Of the 154 nucleotides sequenced, 48 were parsimony informative, which is relatively high for the supposedly conserved 5.8S nuclear rRNA gene.

The phylogenetic analyses showed that the root-derived sequence 1 clustered within the *Acaulospora* lineage, while sequence 2-4 clustered within the *Glomus* group 1 lineage (Fig. 3.13). In addition, repeated Bayesian phylogenetic analysis yielded consistent tree topologies, with the Bayesian consensus topology similar to the strict consensus topology obtained from parsimony analysis, although nodal support based on the Bayesian approach was higher (Fig. 3.13). Nodes supported by Bayesian posterior probability values of $\geq$ 95% and bootstrap values of $\geq$ 70% were considered strongly supported (Fig. 3.13).

3.4. DISCUSSION

Little is known about the AM associations of plants in the CFR, including their functional role in low nutrient ecosystems with acidic soils (Allsopp and Stock 1993a). Concordant with results obtained in this study, a previous study showed that colonization levels of established Fynbos vegetation are usually low (37%) (Allsopp and Stock 1994). Factors contributing to this may be the patchy distribution of AM infectivity in these soils, disturbances such as fire, or the fact that AM colonization of roots is restricted to the first phase of the growth period. The latter case is pertinent for Fynbos vegetation, where efficient uptake of nutrients during the post-fire period is particularly important.

Fire appears to be the major mineralizing agent in Fynbos, replenishing mineral elements held in the above ground phytomass and litter to the soil (Stock and Allsopp 1992). The post-fire environment is characterized by an increased availability of P and N. This availability appears to decrease rapidly as elements become incorporated into plant biomass, as well as being immobilized by decomposer organisms and is thus rapidly returned to pre-fire levels. Therefore, it is an important period for emerging seedlings, and acquisition of nutrients in competition with other organisms would best be mediated by AM fungi (Allsopp and Stock 1993b).

Low levels of available P present in Mountain Fynbos soil (Kruger 1979) would thus maintain high levels of AM colonization to enhance the efficiency of fungal transfer of P to the host (Douds and Schenck 1990). However, as AM fungi are patchily distributed in Fynbos soils, soil inoculum levels may limit initial

colonization, but once colonization occurs, roots rapidly become AM (Allsopp and Stock 1994). For a slow growing species such as *A. betulina*, this initial AM dependence on P-uptake may prevail only until the roots are established. This concurs with the current findings, where the initial growth phase (days 0 to 77) was characterized by high AM colonization levels. During the same period, the increased growth respiration of the AM plants indicated that AM colonization came at increasing C-costs to the hosts (Mortimer *et al.* 2004). This was evident from the lower RGR recorded for the AM plants, compared to the non-AM plants during this period, and concurs with previous studies of AM colonization C-costs (Cavagnaro *et al.* 2003; Mortimer *et al.* 2004). However, the C-costs incurred by the host were balanced by P-uptake.

Aggressive strains of AM fungi may enhance plant P-uptake and growth at low soil P (Graham *et al.* 1996). In our study, total P-uptake was significantly ($P \leq 0.05$) higher in AM than in non-AM plants during the initial stages of growth (days 0 to 77) showing a clear positive AM effect on P accumulation. This was evident in the higher RGR of AM plants in relation to non-AM plants during days 77 to 157, as the enhanced P nutrition may be responsible for the improved growth of AM plants. In addition, the C-costs of the enhanced AM P-uptake were lower in the AM roots during this period, due to the lower P utilization cost as also found in a previous study (Mortimer *et al.* 2004). However, as the percentage AM colonization declined (days 157 to 224) the lower initial P utilization cost of AM plants were increased as AM colonization receded. The absence of the AM benefit for P-uptake may have increased the P-uptake cost for roots, leading to a

C-cost to the plant. In addition, increased AM vesicle formation, which indicated that the symbiont was approaching a stationary phase (Bonfante-Fasolo 1984), may also have resulted in extra C-costs to the plant. Consequently, this all may have lead to a lower P-uptake and RGR in AM plants.

The results of the physiological effects of the indigenous AM associations on *A. betulina* show that this plant from a low nutrient environment is no less reliant on arbuscular mycorrhizae for nutrient acquisition than plants from nutrient rich environments. Despite low growth rates, and therefore low P demand (Chapin 1988), the dependency of these sclerophyllous species on AM fungi for acquiring P from a low nutrient soil (deficient in P) indicates that these species are obligately AM during seedling establishment in their native habitat. However, the exact species level identity of the AM taxa associated with *A. betulina* have, up to now, been completely obscure. Results of the present study have narrowed the identification down to the genera *Acaulospora* and *Glomus*.

Since the AM symbionts do not induce macroscopic changes in the root system (Brundrett *et al.* 1984), microscopic and molecular techniques were used to determine the presence and prevalence of the AM fungi colonizing roots of the host plant. Morphological identification of AM fungi based on staining intensity and intraradical colonization patterns presents intrinsic difficulties, since their intraradical hyphal morphology is almost indistinguishable below the genus level (Helgason *et al.* 1999). Therefore, the molecular method has the potential to provide greater resolution at the infrageneric level, and consequently provides a more robust taxonomy.

However, phenolic substances, polysaccharides, or humic acids in association with DNA obtained from plant tissue or environmental samples have often been reported to inhibit PCR success of these fungi (Di Bonito *et al.* 1995). Furthermore, the DNA obtained from AM fungi needs to be 100 to 500-fold more diluted, compared to the optimal concentration for amplification of genomic DNA of other organisms (Wyss and Bonfante 1993). By diluting the extracts, the inhibitory effect would be reduced, but this leads to a low optimal DNA concentration. Fortunately the method is insensitive to a critical low template concentration, which may also help prevent the amplification of contaminating DNA present. Consequently, dilution of the purified template DNA was required. However, the high concentration of host DNA compared to fungal DNA in roots may limit detection of the latter (Clapp *et al.* 1995). This problem was solved by incorporating fungal specific primers (NS5 and ITS4) in the first reaction of nested PCR.

In the second reaction of nested PCR, a set of PCR primers that target five different families within the phylum Glomeromycota (Redecker 2000) was employed to facilitate amplification of the internal transcribed spacers, 5.8S and partial SSU nuclear rRNA gene fragments from AM colonized roots. These subgroups include the recently discovered deeply divergent lineages of the phylum Glomeromycota, namely species in the genera *Archaeospora* and *Paraglomus*, and species in the genus *Glomus* Group A (Schüβler *et al.* 2001), which could not be detected by previously reported PCR primers (Table 3.1). Furthermore, several of these newly characterized lineages are undetectable

when staining is performed according to standard procedures (Redecker *et al.* 2000). The primers were shown to exclude the majority of other fungal groups, as well as plant DNA. Bands were only detected when amplification was performed with the primer pairs ACAU1660/ITS4 and GLOM1310/ITS4, which indicated colonization by AM fungi belonging to the genera *Acaulospora* or *Entrophospora* and *Glomus*, respectively.

The combination of BLAST searches with subsequent phylogenetic analyses ensured that both the reference sequences obtained from NCBI, as well as the sequences obtained in the study were of glomalean origin. Thus, our phylogenetic analyses should not suffer from distortions caused by sequences from other fungal groups (e.g., ascomycetous fungi) that were mislabelled as glomalean sequences in public databases, a problem affecting various molecular studies of the phylum Glomeromycota (Wubet *et al.* 2003).

In this study, bootstrap values and Bayesian posterior probability estimates for many nodes were low or absent, especially at deeper (internal) nodes in the phylogenetic tree, with only three nodes in the strict consensus topology strongly supported by both parsimony and Bayesian methods of phylogenetic analysis. Possible reasons for the lack of nodal support for many of the nodes may be the small dataset used in the phylogenetic analyses, or that the 5.8S nuclear rRNA gene is too short (154 bp) to provide a robust phylogenetic signal (Redecker *et al.* 1999). This stands in contrast to other studies that found that the relatively short and conserved nuclear 5.8S rRNA gene provided sufficient phylogenetic information to estimate higher level phylogenetic relationships between the

fungal phyla Ascomycota, Basidiomycota, Zygomycota and Glomeromycota, when parsimony (Cullings and Vogler 1998) and Bayesian phylogenetic analyses were performed (Pringle *et al.* 2003; Wubet *et al.* 2003). However, it must be borne in mind that a model of evolution was not assessed by means of Modeltest in the studies performed by Pringle *et al.* (2003) and Wubet *et al.* (2003). Support for deeper nodes was also absent when the neighbor joining method of phylogenetic analysis was performed on the same gene area by both Redecker *et al.* (1999) and Renker *et al.* (2003). The neighbor joining approach to phylogenetic analysis is, however, a poor method of estimating the phylogenetic relationships between organisms (Swofford and Olsen 1990). This may indicate that the rate of molecular evolution of the 5.8S nuclear rRNA gene in the phylum Glomeromycota is different from that of the same gene in other fungi sequenced to date. To improve nodal resolution at deeper nodes for the taxa included in this study, more AM taxa need to be added to the present dataset.

Concerning significantly supported branches, results obtained by parsimony analysis were not in conflict with those inferred from Bayesian analysis, but the latter method yielded a more resolved tree topology. In addition, tree topologies obtained in the phylogenetic analyses are congruent with the tree topology obtained in a previous study, also applying Bayesian analysis (Wubet *et al.* 2003).

Although the assignment of the new sequences to subgroups of the phylum Glomeromycota was possible, most of the new sequences could not be assigned to particular AM species. However, sequences 3 and 4 (Fig. 13) clustered with

reference sequences belonging to *Gl. clarum*, thus revealing the identity of the AM fungi from which sequences 3 and 4 originated. These sequences represent either AM species with yet unpublished full length 5.8S nuclear rRNA gene sequences, or species new to science. Species designation will only be possible after sequencing of the 5.8S nuclear rRNA gene within AM spores, obtained by *in vitro* propagation, using colonized root segments of *A. betulina* originating from a pristine site, as inoculum. *In vitro* propagation is essential for the correct identification of these fungi using both morphological and molecular criteria.

The sensitivity of the molecular method used in this study was gauged by comparison with morphological analysis of stained root segments, which yielded similar results. It is known that plants in the Rutaceae form both or intermediate types of the *Paris* and *Arum*-type hyphae (Smith and Smith 1997). Some root samples obtained from the field site contained intraradical spores, characteristic of *Gl. clarum* and *Gl. intraradices.* These two species differ from other glomalean species because they sporulate primarily within the host root, rather than in the soil (Douds and Schenck 1990). Arbuscular mycorrhizal fungi may form spores inside the root by forming temporary hyphae, which re-infect the host to obtain the C required for sporulation, because the mycelia of the AM fungi cannot take up C from any source other than the host root (Gadkar and Adholeya 2000). Further supportive evidence for the identity of the AM fungi obtained is the fact that AM fungi such as those in the genera *Acaulospora* and *Glomus* are widely reported in acidic soils, such as the soil type investigated in this study (Clark 1997).

3.5. CONCLUSION

The P nutritional benefits of the symbiosis coincided with the rapid phase of AM development and the early stages of host growth. This may be typical of a sclerophyllous seedling in a post-fire ecosystem, where the plant takes advantage of pulsed nutrient flushes (Allsopp and Stock 1993b), where after the host's reliance on AM fungi for P nutrition declines. This thus ensures an adequate supply of P to support growth during periods when nutrients are unavailable (Chapin 1988). The AM taxa (*Glomus* and *Acaulospora*) responsible for this symbiosis may therefore be adapted to this nutrient-limited host reliance. However, it must be borne in mind that the AM specific primers employed in the second reaction of nested PCR only amplify nuclear rRNA sequences (partial SSU; full length ITS1, 5.8S, and ITS2) of six of the known clades within the phylum Glomeromycota, with the exception of the *Glomus vesiforme* clade (Wubet *et al.* 2003). Therefore, our results should only be considered as a survey of these clades within the roots of *A. betulina* under the specific experimental conditions used, with the possibility that other AM fungi might have been present within the roots of these plants. As molecular tools with greater resolving power are being developed, the full AM diversity associated with *A. betulina* should be revealed in future.

3.6. FIGURES AND TABLES

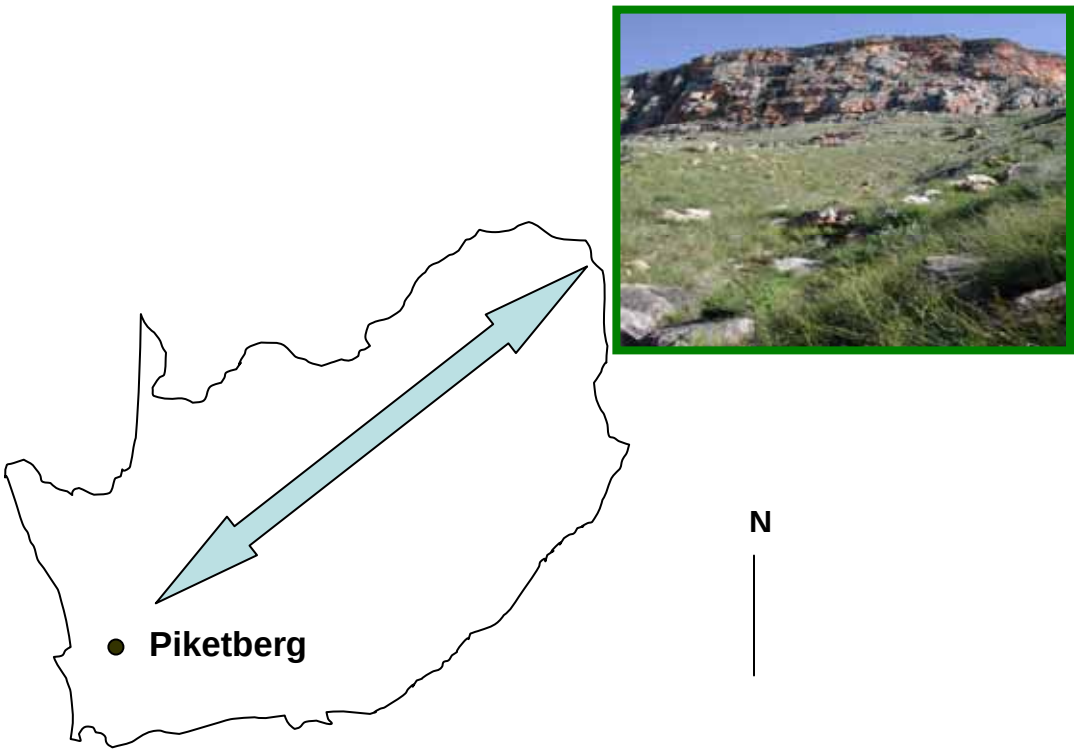

FIG. 3.1. Map indicating the sampling site (arrow). The insert is a photograph of the sampling site at the time of sampling, taken during July 2003.

FIG. 3.2. Slides containing 25 stained *Agathosma betulina* root segments each. These were prepared for determination of percentage root length colonized by arbuscular mycorrhizal fungi.

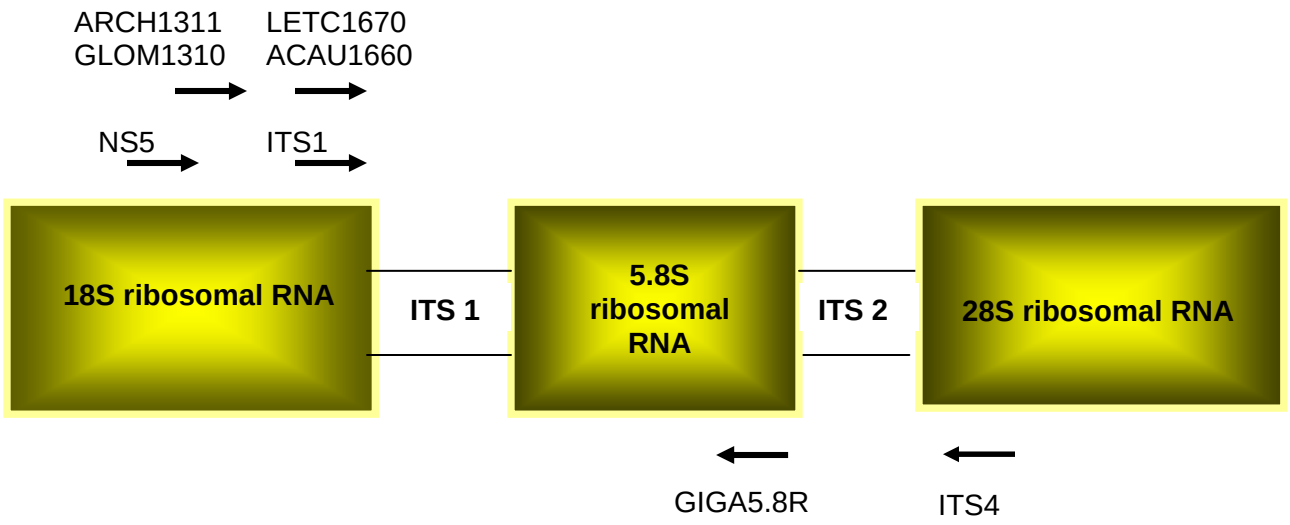

FIG. 3.3. Schematic representation of the nuclear ribosomal RNA gene cluster and the relative annealing sites of the forward arbuscular mycorrhizal (AM) specific- (ARCH1311, GLOM1310, LETC1670, and ACAU1660), reverse AM specific- (GIGA5.8R) and universal fungal primers (NS5, ITS1, and ITS4) used in amplification reactions in this study. The figure is not drawn to scale and is based on the studies of Redecker (2000). Sequences of the primers are listed under the heading: "3.2.15. Amplification of ribosomal RNA (rRNA) genes". S, Svedberg sedimentation coefficient; ITS, internal transcribed spacer.

FIG. 3.4. Light micrograph of healthy arbuscular mycorrhizal (AM) spores (arrows) on a cellulosic membrane filter (0.22 µm pore size). These AM spores were isolated from the rhizosphere soil of pristine stands of *Agathosma betulina* plants by means of wet sieving and decanting, followed by sucrose density gradient centrifugation (Brundrett 1994c).

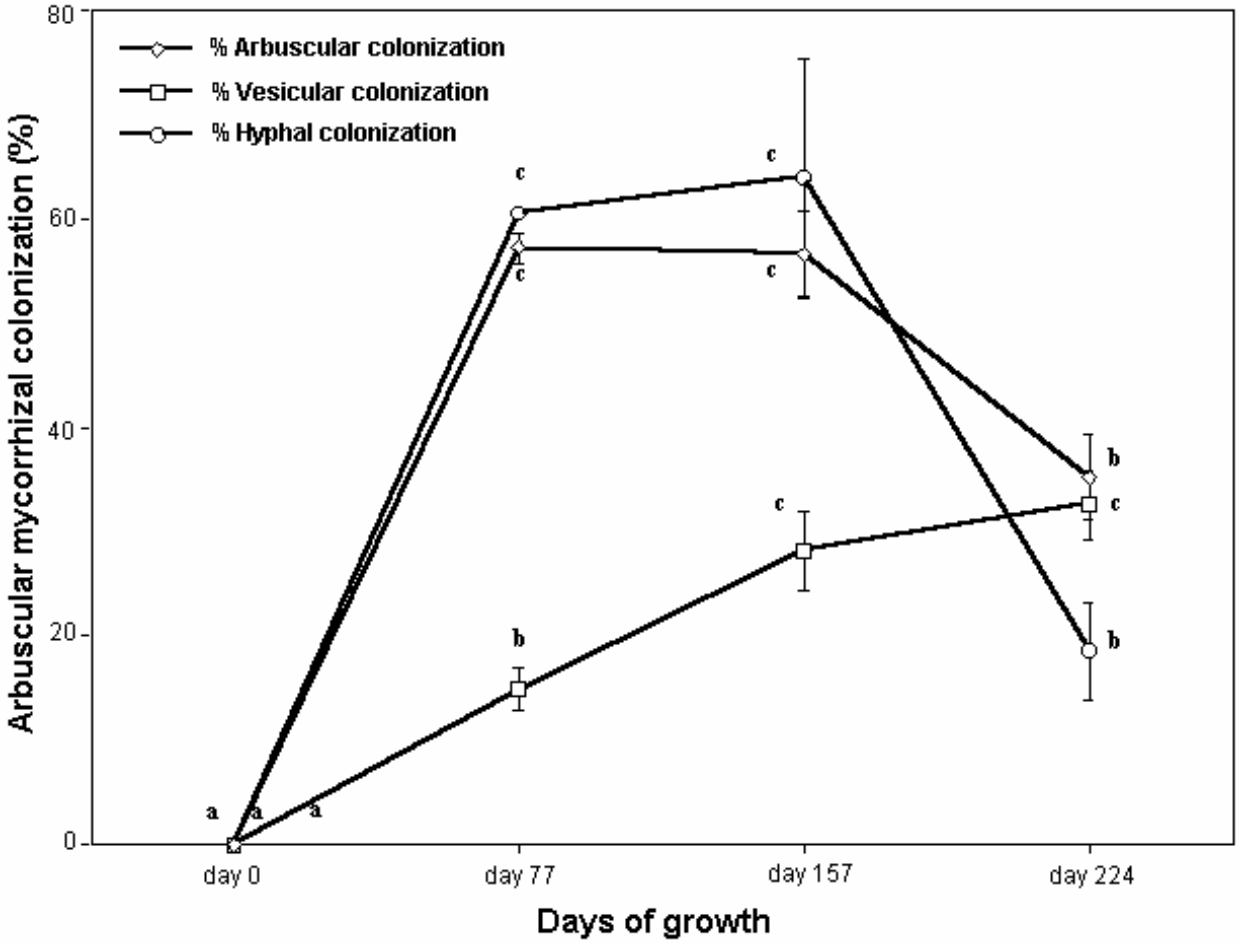

FIG. 3.5. Percentage (%) root length colonized by arbuscular mycorrhizal fungi (% arbuscular-, % vesicular-, and % hyphal colonization) belonging to the genera *Acaulospora* and *Glomus*. *Agathosma betulina* plants were grown under glasshouse conditions and harvested at day 77, 157 and 224. The differences between harvests (n = 4) were separated using a post hoc Student Newman Kuels, multiple comparison test ($P \leq 0.05$). Statistically significant differences ($P \leq 0.05$) between each harvest are indicated by different letters. Error bars indicate the standard error of the mean for each treatment (n = 4).

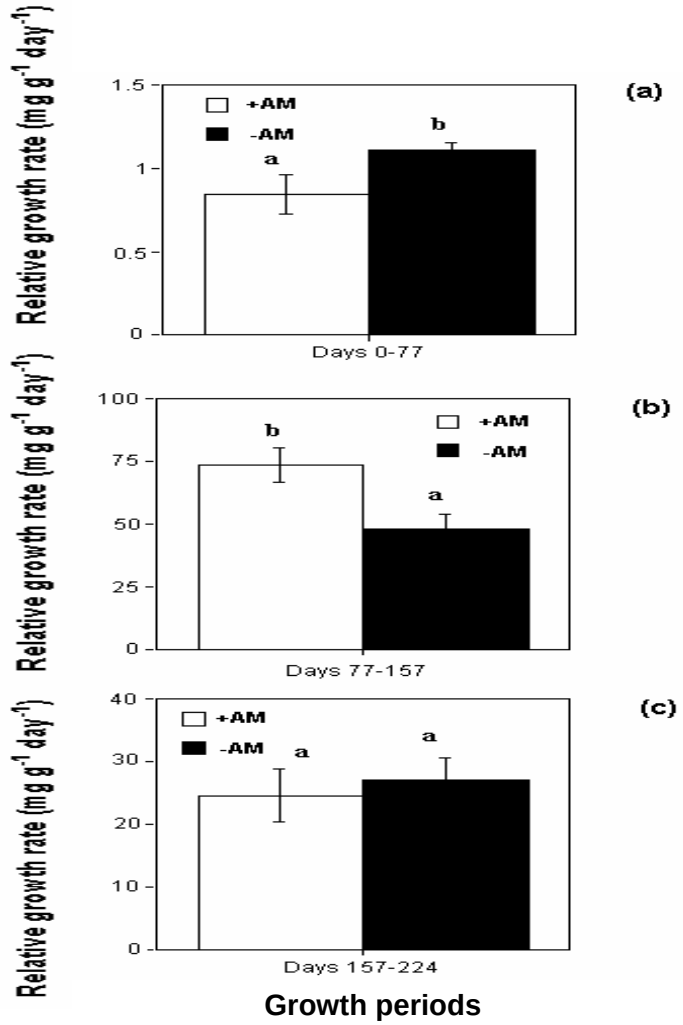

FIG. 3.6. Relative growth rate (mg g^{-1} day^{-1}) for (a) the growth period 0-77 days, for (b) the growth period 77-157 days and for (c) the growth period 157-224 days, of glasshouse-cultivated *Agathosma betulina* plants colonized by arbuscular mycorrhizal (AM) fungi belonging to the genera *Acaulospora* and *Glomus.* Plants were harvested at day 77, 157 and 224. Differences between inoculated (+AM) and non-inoculated (-AM) plants were determined by *t*-test for independent samples by groups. Statistically significant differences ($P \leq 0.05$) between inoculated and non-inoculated plants for each growth period are indicated by different letters. Error bars indicate the standard error of the mean for each treatment ($n = 4$).

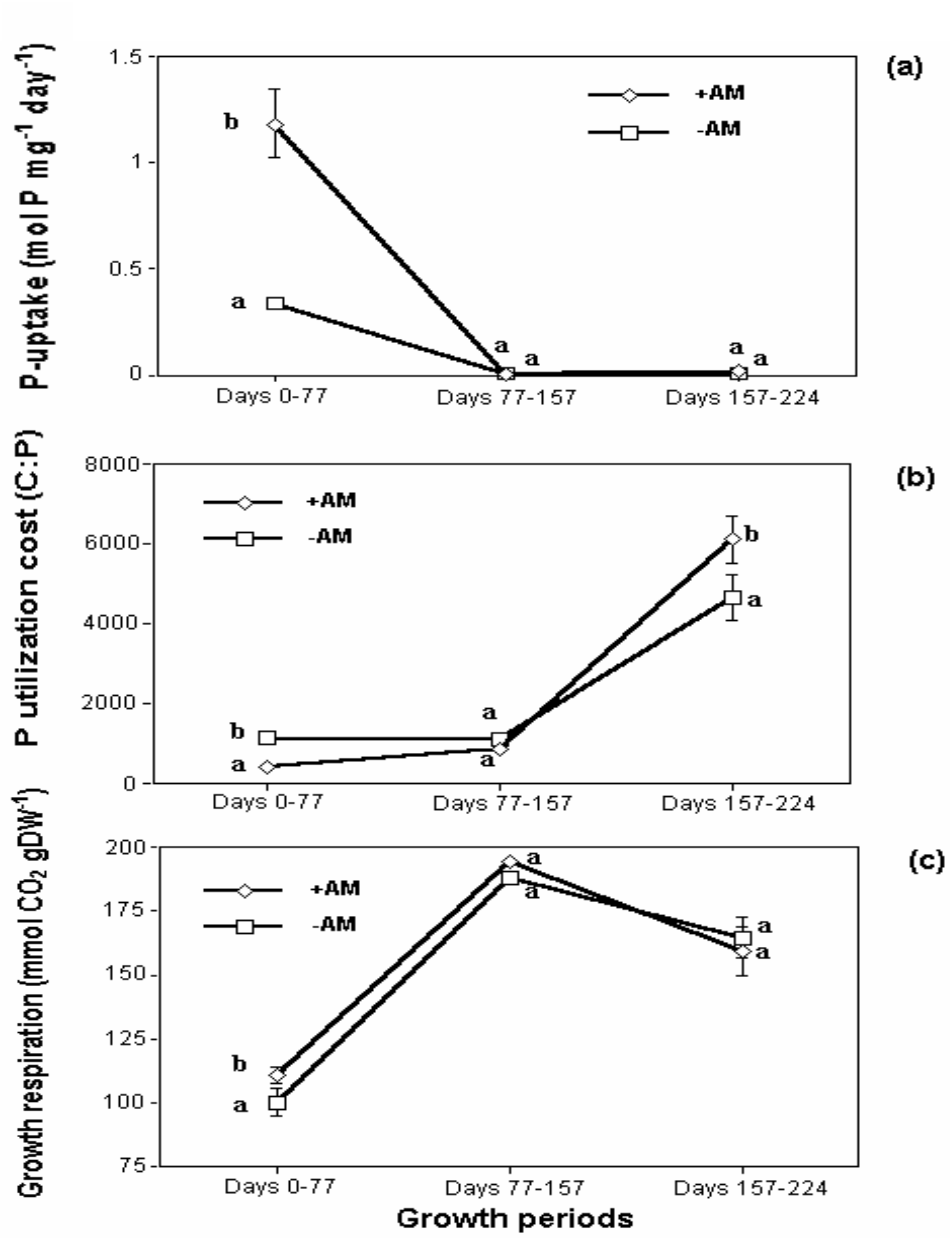

FIG. 3.7. (a) P-uptake (mol P mg^{-1} day^{-1}), (b) P utilization cost (C:P), and (c) growth respiration (mmol CO_2 gDW^{-1}), of glasshouse-cultivated *Agathosma betulina* plants colonized by arbuscular mycorrhizal (AM) fungi belonging to the genera *Acaulospora* and *Glomus.* Plants were harvested at day 77, 157 and 224. Differences between inoculated (+AM) and non-inoculated (-AM) plants were determined by *t*-test for independent samples by groups. Statistically significant differences ($P \leq 0.05$) between inoculated and non-inoculated plants for each growth period are indicated by different letters. Error bars indicate the standard error of the mean for each treatment ($n = 4$).

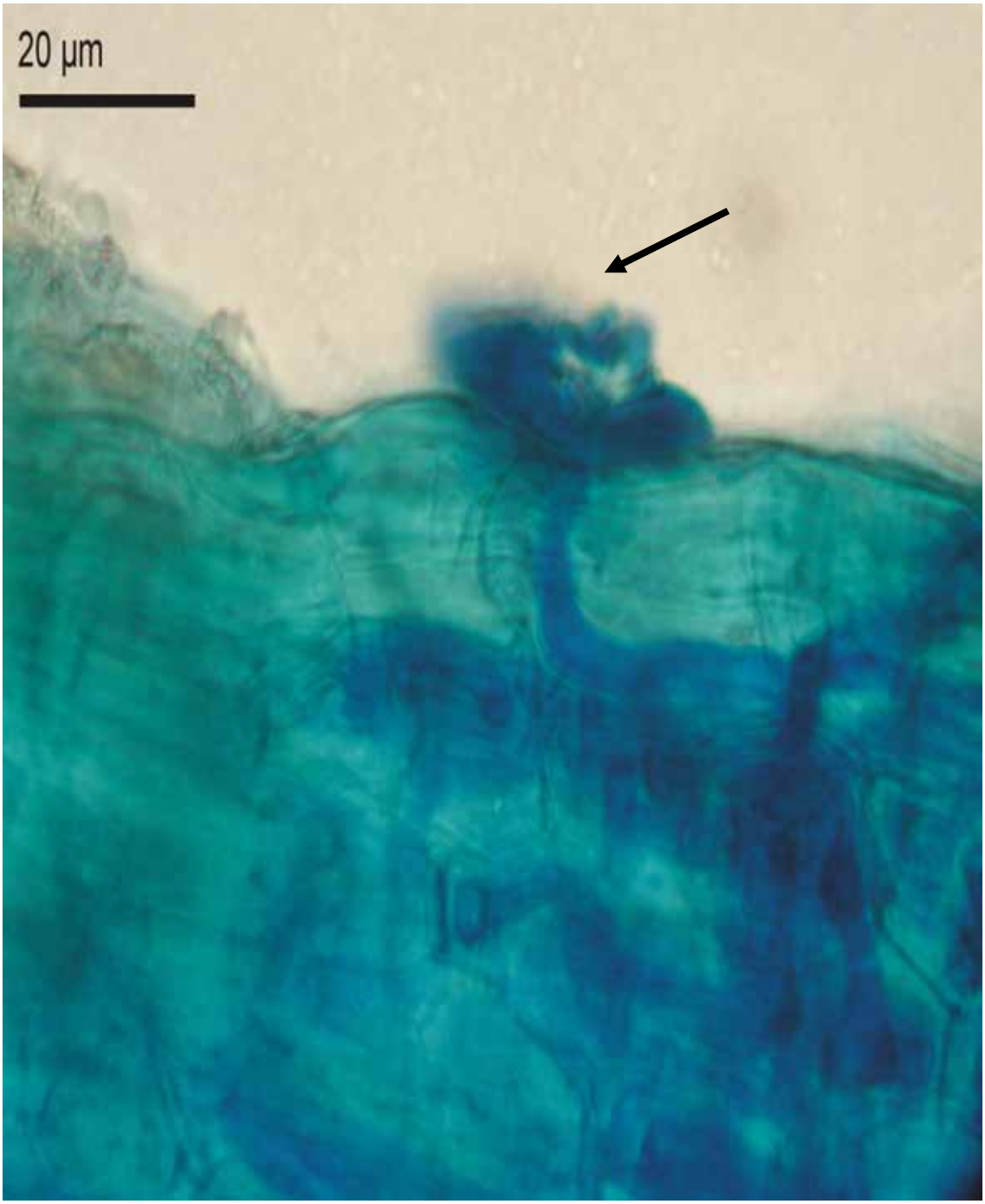

FIG. 3.8. Light micrograph of an aniline blue stained (Brundrett 1994a) *Agathosma betulina* root segment obtained from pristine stands of this plant, showing an appressorium (arrow) formed between the root and soil interface.

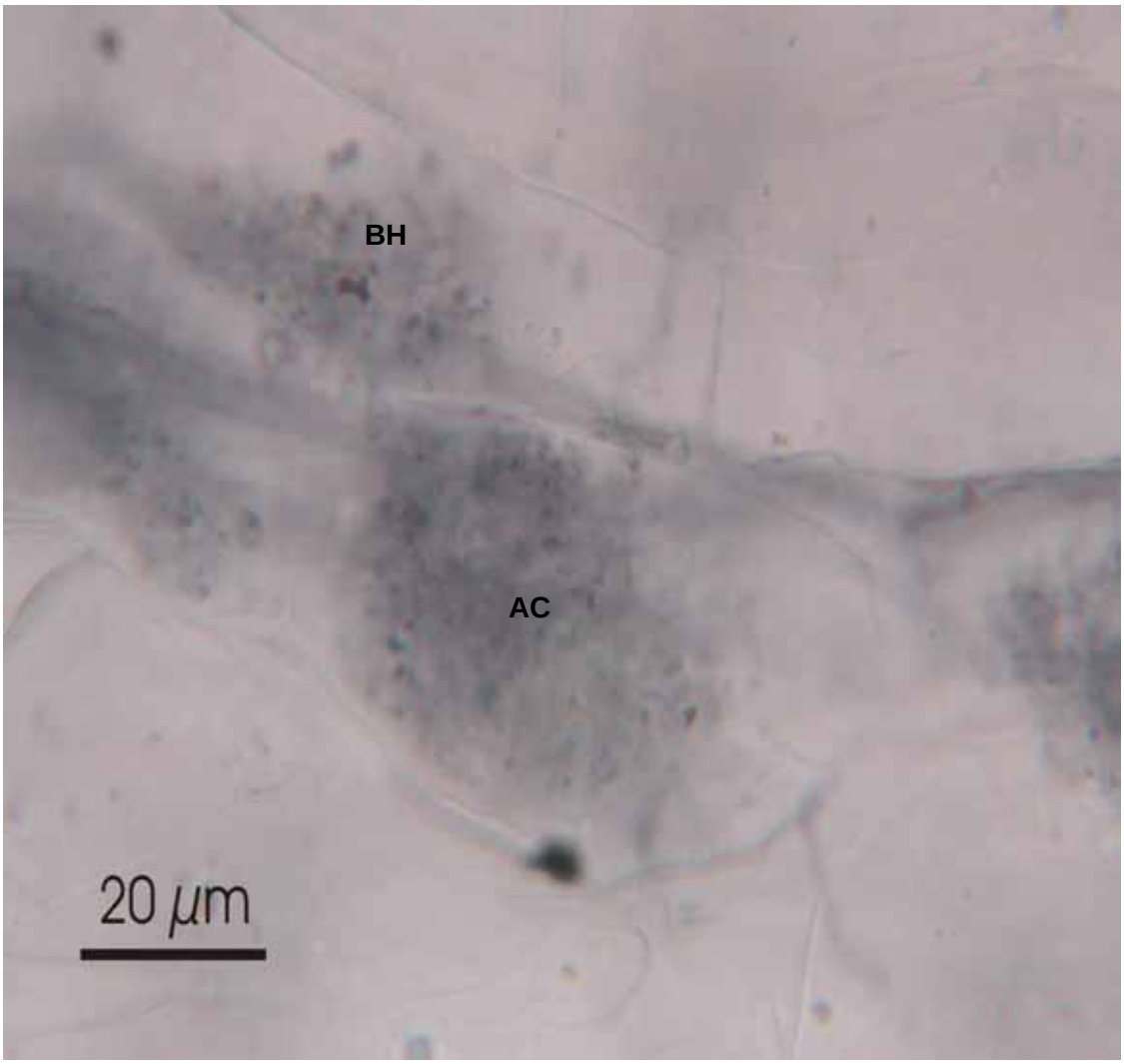

FIG. 3.9. Light micrograph of arbuscules characteristic of the genus *Glomus* in a chlorazol black E stained (Brundrett 1994a) *Agathosma betulina* root segment obtained from 157 day old pot-cultured plants. The arbuscules proliferate from a cylindrical, sometimes swollen trunk hypha of 5 μm in diameter and end in a proliferation of fine branched hyphae (BH), < 1 μm in diameter (Brundrett *et al.* 1996). Large arbuscular clumps (AC), formed by the aggregation of smaller ones (Bonfante-Fasolo 1978), were also noted.

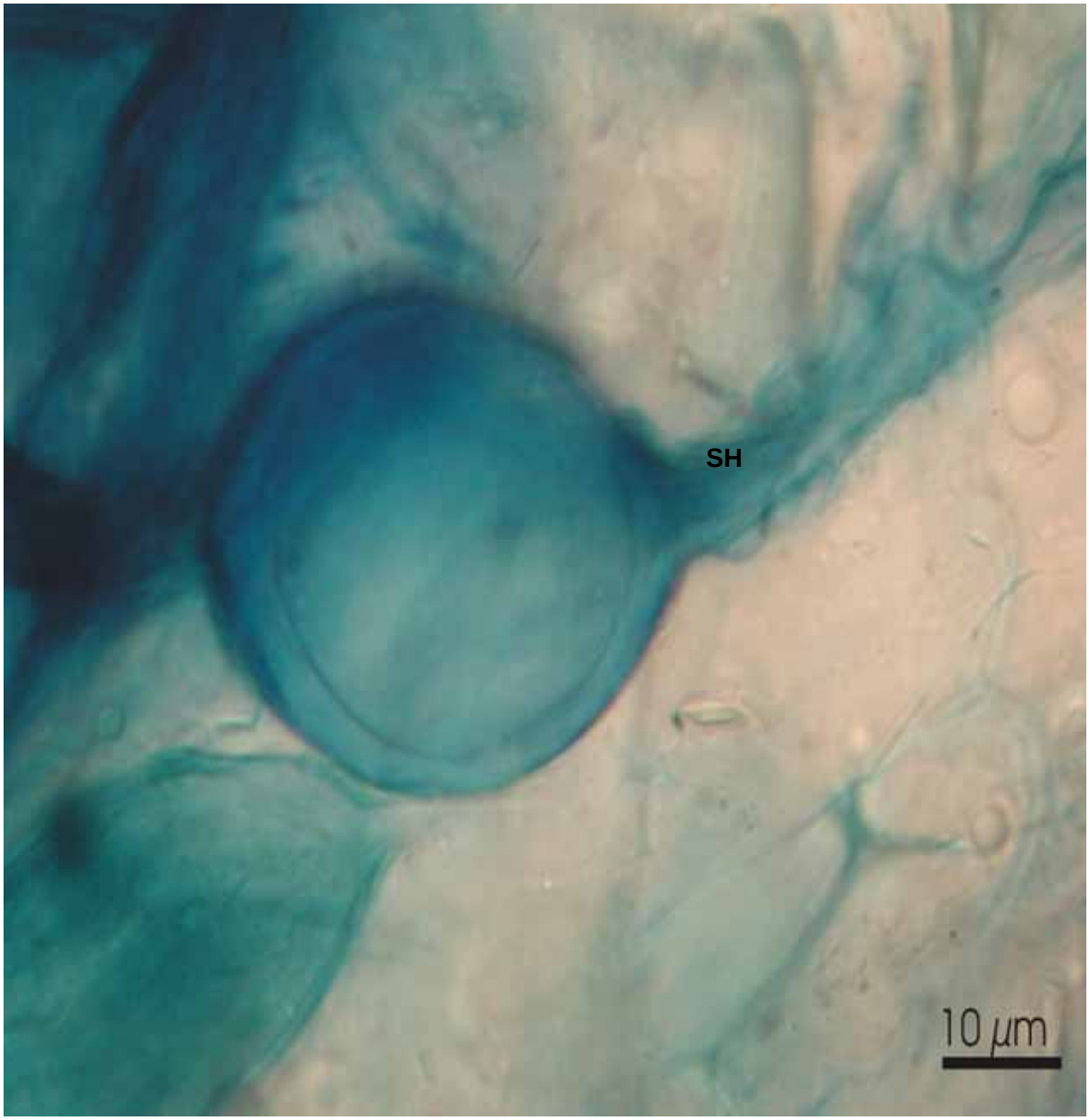

FIG. 3.10. Light micrograph of a thin mount made from a squashed aniline blue stained (Brundrett 1994a) field sampled root segment of *Agathosma betulina*, showing a single intraradical arbuscular mycorrhizal spore characteristic of *Glomus clarum*. The spore is attached to an intracellular subtending hypha (SH).

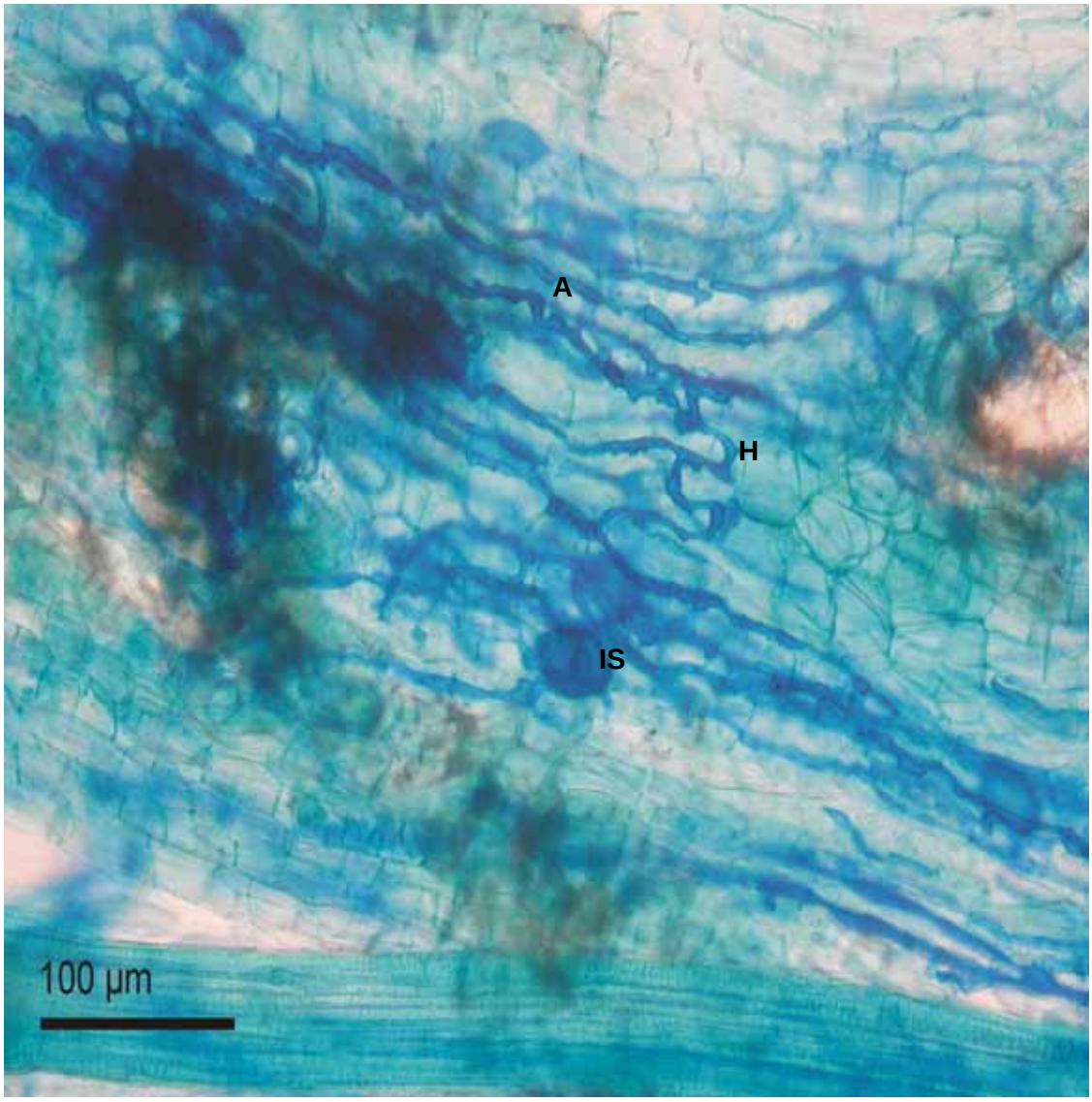

FIG. 3.11. Light micrograph of *Arum*-type (A) intercellular hyphae in a field sampled *Agathosma betulina* root segment stained with aniline blue (Brundrett 1994a) and colonized by the genus *Glomus*. *Arum*-type intercellular colonization hyphae of 1.5 to 4 µm wide grow parallel to each other and to the root axis, and interconnect via acute-angle branched hyphae, often referred to as "H" (H) connections (Brundrett *et al.* 1996). Intraradical spores (IS) were usually formed terminally on branched hyphae within or between cortical cells, near entry points.

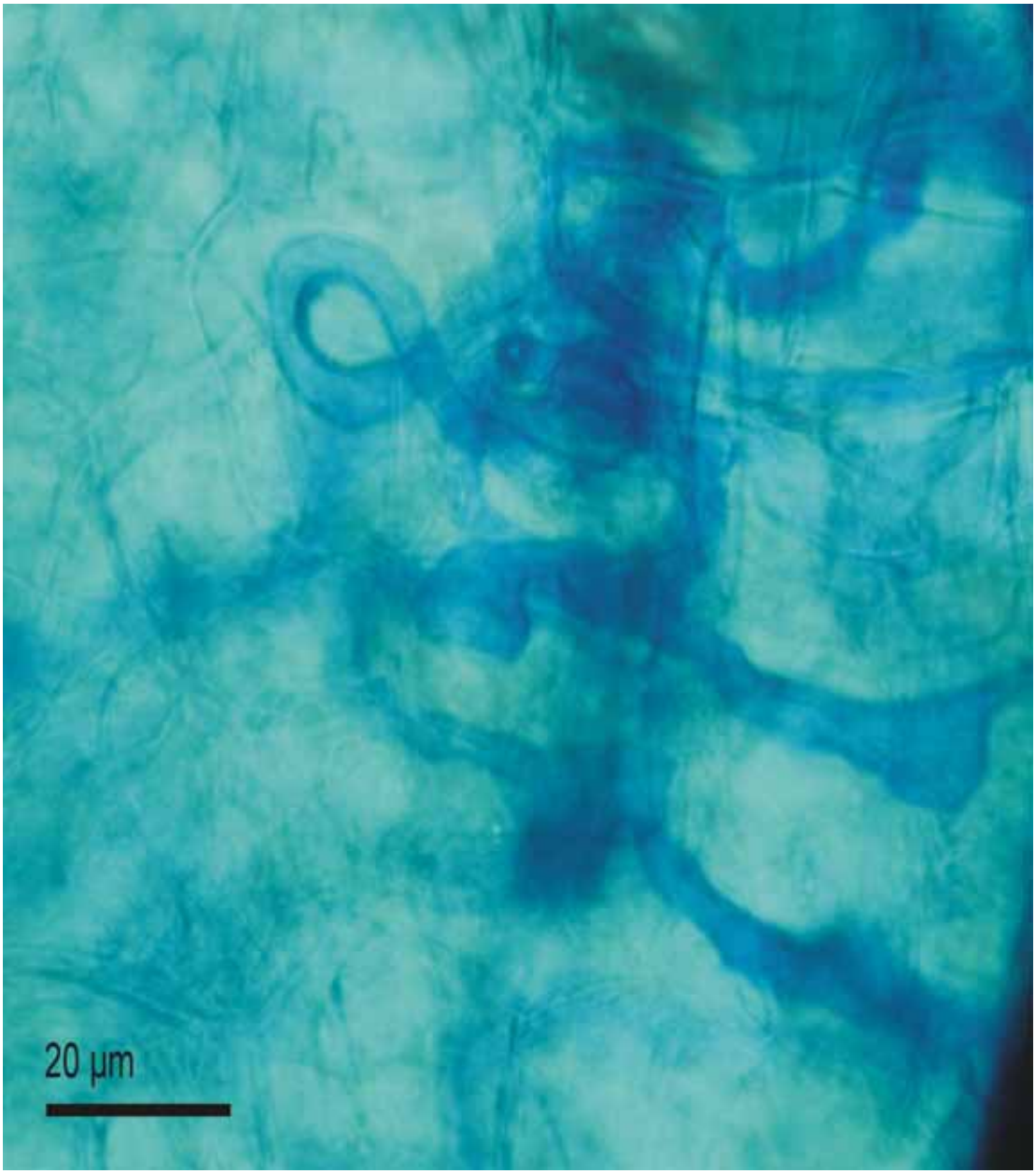

FIG. 3.12. Light micrograph of *Paris*-type intracellular hyphae near entry points in a field sampled *Agathosma betulina* root segment stained with aniline blue (Brundrett 1994a) and colonized by the genus *Glomus*.

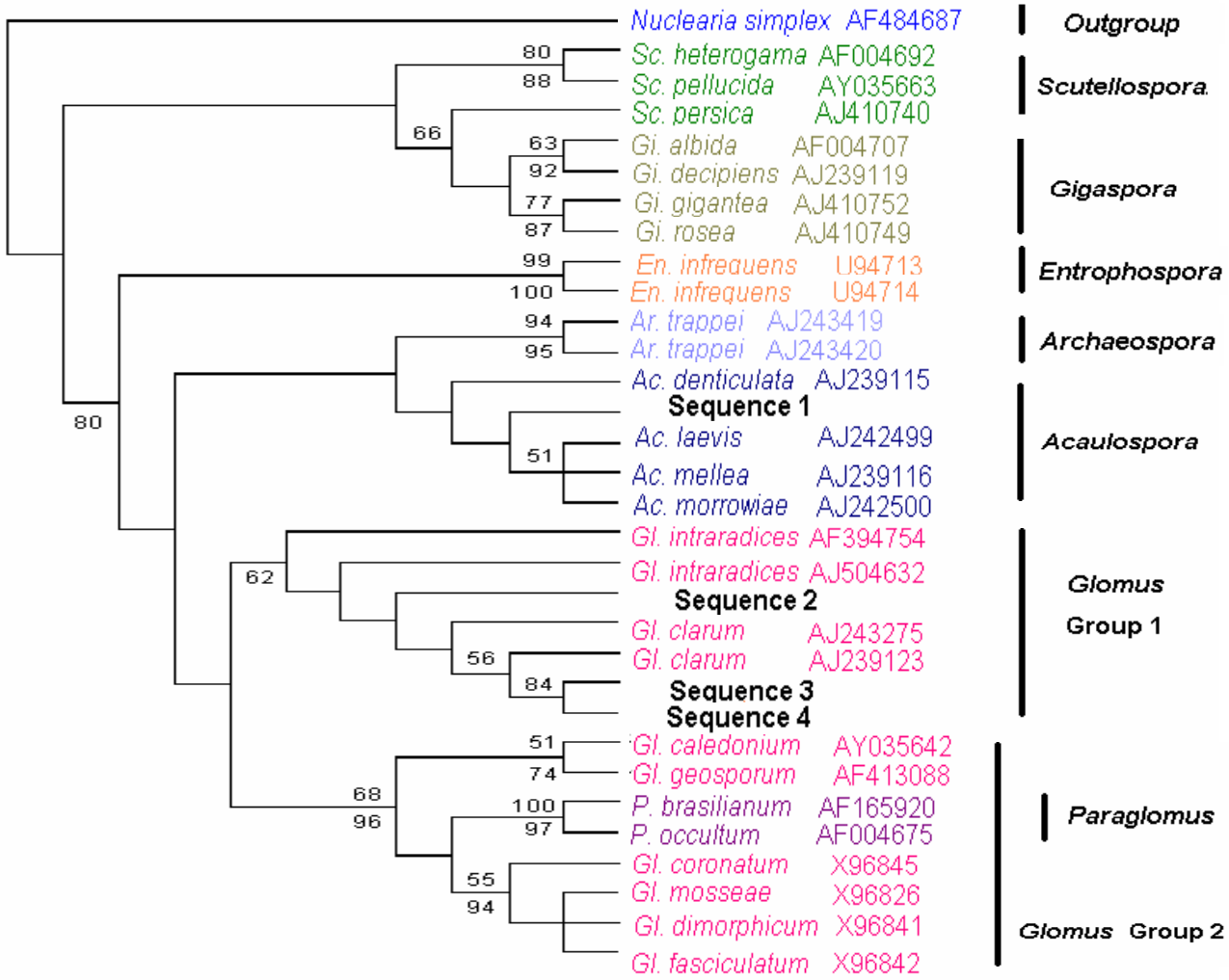

FIG. 3.13. Strict consensus tree of 14 trees based on full length 5.8S (S, Svedberg sedimentation coefficient) nuclear ribosomal RNA (rRNA) gene sequences of the phylum Glomeromycota. Numbers above branches refer to bootstrap percentages. Numbers below branches are Bayesian posterior probability estimates, which are expressed as percentages. Only bootstrap and posterior probability support over 50% are shown. The topology was rooted with *Nuclearia simplex*. Bold names are representatives of arbuscular mycorrhizal 5.8S nuclear rRNA gene sequences obtained during this study. Sc., *Scutellospora*; Gi., *Gigaspora*; En., *Entrophospora*; Ar., *Archaeospora*; Ac., *Acaulospora*; Gl., *Glomus*; and P., *Paraglomus*.

TABLE 3.1. Specificity of arbuscular mycorrhizal specific primers (Redecker 2000) employed in this study by means of nested PCR [a].

Arbuscular mycorrhizal species	ARCH 1311	ACAU 1660	LETC 1670	GLOM 1310	GIGA 5.8R
Archaeospora gerdemannii	+	-	-	-	-
Archaeospora trappei	+	-	-	-	-
Paraglomus occultum	+	-	-	-	-
Paraglomus brasilianum	+	-	-	-	-
Acaulospora laevis	-	+	-	-	-
Acaulospora lacunosa	-	+	-	-	-
Acaulospora morrowiae	-	+	-	-	-
Acaulospora mellea	-	+	-	-	-
Acaulospora spinosa	-	+	-	-	-
Entrophospora colombiana	-	+	-	-	-
Glomus etunicatum	-	-	+	-	-
Glomus claroidium	-	-	+	-	-
Glomus geosporum	-	(+)	-	+	-
Glomus caledonium	-	(+)	-	+	-
Glomus clarum	-	-	-	+	-
Glomus sinuosum	-	-	-	+	-
Glomus coremioides	-	-	-	+	-
Gigaspora decipiens	-	-	-	-	+
Gigaspora albida	-	-	-	-	+
Gigaspora rosea	-	-	-	-	+
Scutellospora heterogama	-	-	-	-	+
Scutellospora pellucida	-	-	-	-	+

[a] " + " Signs represent positive results to be obtained in nested PCR when the corresponding arbuscular mycorrhizal (AM) taxa are present, while " - " signs represent negative results to be obtained in nested PCR when the corresponding AM taxa are present. " (+) " Signs indicate potential positive results to be obtained in nested PCR when the corresponding AM taxa are present.

TABLE 3.2. Statistics for the parsimony based phylogenetic analysis performed on 5.8S nuclear ribosomal RNA gene sequences.

Number of heuristic iterations	1000
Number of characters (characters unordered and equally weighted)	154
Number of constant characters	80
Number of parsimony uninformative characters	26
Number of parsimony informative characters	48
Number of trees found	14
Consistency index	0.699
Retention index	0.814

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