Role of Fatty Acid Techniques in Studying AM Fungi

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Abstract

The main aims of this project were to determine if fatty acid analysis could be used to (1) detect and quantify AM fungi in the roots of host plants or in colonised soils and (2) to differentiate between AM fungi at the genera and species level. Two techniques of fatty acid analysis were used; one based on analysis of fatty acid methyl esters (FAME) following extraction of total lipids (cellular and non-cellular lipids) from soil and root samples, and the other based on analysis of phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA), following extraction and fractionation of cellular lipids. The two techniques were compared in two experiments (reported in Chapters 3 and 4) where FAME analysis was found as good as PLFA / NLFA analysis for the detection of specific fatty acids. Because of the FAME was simpler and could be used for fatty acid analysis of spores, it was used for all experiments reported in the subsequent chapters.

Fatty acid 16:1ω5c was confirmed as a general biomarker for species of AM fungi. It could be used reliably to detect the presence of AM fungi in plant roots and soil. In addition, several other fatty acids, namely 18:1ω9c, 20:1ω9c, 20:2ω6c and 22:1ω9c were detected specifically in the spores of *Gigaspora* species suggesting their use as possible biomarkers for the detection of species of this genus. However, the *Gigaspora* species used in this work could not be differentiated on the basis of FAME analysis of spores.

A positive correlation was found between the amount of 16:1ω5c detected in soil and plant roots colonised by different AM fungi in pot experiments and two conventional methods of measuring AM infectivity (number of spores, and % colonisation of roots). This provided good evidence for the potential use of 16:1ω5c to estimate AM infectivity in soil and plant roots in controlled experimental systems. A similar strong positive correlation was also observed between the AM infectivity of 3 field soils (measured by spore counts and % root colonisation by 4 different host plants) and the amount of 16:1ω5c detected in soil FAME profiles. This suggests that 16:1ω5c could be used to detect and quantify AM fungi in field soils.

The amount of 16:1ω5c in individual spores of different AM species was determined on two separate occasions. The amount detected varied with different

AM species, was unrelated to spore weight and could be used to estimate the proportion of 16:1ω5c detected in soil samples associated with AM fungal spores. Addition of different combinations and quantities of spores of AM species to soil gave the expected ratios of marker fatty acids (including those associated with *Gigaspora* species) in the soil FAME profiles. The spores of *Glomus coronatum* and *G. mosseae* contained larger quantities of 16:1ω5c than the spores of *Gigaspora margarita* or *Scutellospora calospora*.

An experiment was designed to follow changes in amount of 16:1ω5c detectable in different components of the *G. coronatum* during the development of the plant – mycorrhizal association. This involved separation of colonised plant roots from soil containing AM fungal hyphae only. The amount of 16:1ω5c by FAME or PLFA analysis of the soil associated with the plant roots showed a trend comparable to the sigmoid growth pattern of spore formation and root colonisation by the mycorrhiza. The concentration of 16:1ω5c in the soil associated with the plant roots was greater than that in the soil free of roots.

A number of experiments were conducted to validate fatty acid analysis to mycorrhizal studies. This included analysis of the roots of a range of plants that normally form mycorrhizal associations with AM fungi. The roots of these plants were shown not to contain fatty acids (including 16:1ω5c) known to be associated with AM fungi.

Publications

Journal Articles:

Madan R., Pankhurst C., Hawke B., Smith S. (2002). Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. **Soil Biology and Biochemistry** 34, 125-128.

Conference Articles:

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R. Madan, S.E. Smith, C. E. Pankhurst, B. Hawke and F. Andrew (2000). The role of fatty acids in detection of mycorrhizal fungi and estimation of propagule biomass. **Third International Congress on Symbiosis, 13-19 August, Phillips-Universität Marburg, Germany.**

DECLARATION

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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November 2002

Signed

Rajni Madan

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Finally our wait is over!!

Chapter 1: Literature Review

1. Introduction

1.1 AM Mycorrhiza

Mycorrhizas are symbiotic associations between plant roots and certain soil fungi. The predominant type is vesicular-arbuscular mycorrhiza also known as arbuscular mycorrhiza (AM) formed by fungi from the order Glomales of division Zygomycotina of Eucaryota. About 130 species have been described in the Glomales (Walker 1992; Walker & Trappe 1993) including a few non-mycorrhizal species. Mycorrhizal fungi colonise most plant taxa (about 80%, Smith & Gianinazzi-Pearson 1988) and Glomalean fungi are found in all geographic regions. The relationship between fungi and host is obligate for the fungi, which only grow vegetatively and complete their life cycles in symbiosis.

1.1.1 Significance of AM Mycorrhiza

The beneficial effects of AM mycorrhizal fungi to their host plants and to the environment have been well documented (Smith & Gianinazzi-Pearson 1988; Smith & Read 1997). Mycorrhizal fungi are of benefit to plants in many ways. The growth enhancement observed in highly dependent host crops eg. linseed (Dugassa *et al.* 1996), soy bean (Abdelfattah 1997), sunflower (Chandrashekhar *et al.* 1995), sugarcane (Wang *et al.* 1992; Magarey & Bull 1997), and corn (Kurle & Pfleger 1996), suggests that their relationship is in fact mutualism (Bethlenfalvay 1992). A study of correlations between plant and soil traits indicated that the interactions within the plant-soil system, were mediated by the AM fungi (Schreiner *et al.* 1997). The special haustoria-like structures (arbuscules or coiled hyphae) that interface with the host cytoplasm in root cortical cells, provide increased surface area for metabolic exchanges between the host and fungal partners (Linderman 1992).

Mycorrhizal fungi also interface with the surrounding rhizosphere by producing extra-radical hyphae, which extend into the soil and increase the potential of the root system for nutrient and water absorption. These hyphae have been demonstrated to have the potential to improve mineral absorption (eg. P and Zn) by plants, thus contributing to increased crop yields. In one study, the external hyphae

of the AM fungi *Glomus fasciculatum* and *G. mossae* were able to acquire and transport N and P in relatively dry soil, contributing to increased biomass of their host plants (Tobar *et al.* 1994). By allowing root-induced changes in the rhizosphere by organic acid exudation, AM fungi increase the spatial availability of mineral nutrients in the soil particularly in nutrient-poor soils, thus enhancing host plant growth (Marschner 1996). Azcon Aguilar *et al.* (1993) used isotopic evidence to show that AM fungi had the capacity to use less available forms of N in the soil. The extra-radical hyphae of AM fungi also play an important role in the stabilisation of soil aggregates (Miller & Jastrow 1992; Tisdall 1991; Tisdall *et al.* 1997), and may therefore contribute to improved aeration and reduced erosion of soils.

Mycorrhizal plants exhibit changes in some physiological and biochemical attributes compared to non-mycorrhizal plants. For example, mycorrhizal plants show differences in the concentration of growth regulating substances eg. auxins, gibberellins and cytokinins and may show an increased rate of photosynthesis (due to increased uptake of minerals by the roots) compared to non-mycorrhizal plants. Mycorrhizal plants may also show changes in the structural and biochemical aspects of root cells, which affect membrane permeability and thus the quality and quantity of root exudation. Altered exudation changes the composition of microorganisms in the rhizosphere soil (Linderman 1992).

More recently mycorrhizas have been shown to enhance the infection of plant roots by endophytic nitrogen fixing bacteria (Boddey *et al.* 1991; Wang *et al.* 1992; Muthukumarasamy *et al.* 1994; Wang *et al.* 1995; Chanway 1997). Muthukumarasamy *et al.* (1994) reported that the nitrogen-fixing bacteria, *Acetobactor diazotrophicus* and *Herbaspirillium seropedicae*, when combined with AM fungi as a biofertiliser, were able to reduce the need for mineral N fertiliser by 50% and increased crop productivity by 5-7 t/acre. Others have reported on the potential of AM fungi as biofertilisers and bioprotectors and how this could result in a reduction in the use of chemical fertilisers and pesticides, particularly in horticulture (Azcon Aguilar & Barea 1997). Selection of specific AM fungal species for specific crops has been suggested as a tool to improve agricultural management practices (Hoffman & Carroll 1995).

1.1.2 Stages of Development of Mycorrhiza

Root colonisation by AM fungi can be initiated by a range of propagules, including spores and infected root pieces (Smith & Smith 1981; Smith & Read 1997). The propagules develop a vegetative mycelium that grows towards the plant root and produces appressoria upon coming into contact with the host plant root surface. The appresorium produces hyphae, which initiate the colonisation of roots by forming intercellular hyphae, coils, vesicles and arbuscules (Carling & Brown 1982; Bonfante-Fasolo & Perotto 1992). There are some variations in the final structural attributes of the mycorrhiza that is influenced both by the host plant species and the fungus (Brundrett *et al.* 1985; Smith & Smith 1996).

The growth of vegetative mycelium towards the plant root appears to be influenced by some form of chemical signal from the host plant (Gemma & Koske 1988). Plant flavonoid compounds have been found to stimulate hyphal growth in vitro, and may act as molecular signals in the symbiosis (Gianinazzi-Pearson *et al.* 1989; Siqueira *et al.* 1991; Poulin *et al.* 1997).

The time taken by the fungus to develop infective hyphae on the root surface may be as little as one day (Brundrett *et al.* 1985). Within the cortex these hyphae develop arbuscules 36-48h after the initiation of the infection (Cox & Sanders 1974; Brundrett *et al.* 1985; Giovannetti & Citernesi 1993). The arbuscules are considered the main organs involved in solute exchange between fungus and plant cells (Smith & Smith 1981; Smith & Read 1997). They grow to their maximum size in 4-5 days (Toth & Miller 1984; Alexander *et al.* 1989), occupying about 40% of the cell space, after which they start degenerating (Toth & Miller 1984). The rate of growth of intercellular hyphae has been estimated at 0.13-1.22 mm per day depending on the method of estimation, the host plant species and the environmental conditions (Smith & Walker 1981; Walker & Smith 1984; Brundrett *et al.* 1985; Bruce *et al.* 1994). Extensive growth of external mycelium occurs only after appressoria have been formed (Mosse & Hepper 1975; Hepper 1981). Vesicles, which store large amounts of lipids and also contain many nuclei, appear after the infective hyphae have matured. Although mainly considered to be storage organs, the vesicles may also function as propagules either within root fragments or when isolated from roots. The existence of a multi-layered wall structure, similar to that present in AM fungal spores, is the basis of this assumption (Biermann & Linderman 1983). After

colonisation of the root cells, hyphae external to the root cells, grow and colonise the soil in the rhizosphere. These external hyphae play a major role in nutrient absorption.

Reproduction in AM mycorrhiza is normally asexual although sexual structures have been observed in *Gigaspora decipiens* (Tommerup 1988; Tommerup & Sivasithamparam 1990). A reassortment of nuclei, exchange of mitochondria, extranuclear DNA, RNA and protein molecules has been reported to occur between compatible fungi after anastomosis during asexual reproduction. In contrast, the complete absence of nuclear division during in vitro development of mycorrhiza, has been reported by Burgraaf & Beringer (1989). However, this has not been confirmed and the complete life cycle of AM fungi is poorly understood. Little is known about the development of new spores and what conditions trigger spore formation. Only a few studies have been carried out on nuclear division (Bonfante-Fasolo & Perotto 1992; Bécard & Pfeffer 1993; Bianciotto & Bonfante 1993) and the occurrence of positional and structural changes in the nuclei in arbuscules and host plant cells during the establishment of the symbiosis (Balestrini *et al.* 1992).

1.1.3 Detection of Mycorrhizal fungi

Because of the importance of AM fungi in both natural and agricultural ecosystems, considerable effort has been directed towards the development of methods of detection and identification of AM fungi in the soil and inside plant roots. However, detection and identification of AM fungi in soil or plant roots is inherently difficult because of their obligate association with plants. Currently, taxonomic description and identification of AM fungi is based mainly on spore characteristics, although it is possible to identify a few species on the basis of their root colonisation pattern (Abbott 1982; Brundrett *et al.*1984). Spores used for identification purposes need to be raised in pot cultures, because field-collected material is frequently damaged. However, there are many limitations associated with spore identification including the fact that some AM fungi may not produce identifiable spores (Johnson 1977; Morton 1988; McGee 1989).

Molecular and biochemical approaches are increasingly being used to detect, identify and characterise AM fungal species. These methods mainly include isozyme analysis (Hepper & Sen 1986; Morton 1987; Rosendahl & Sen 1992), protein profiles

(Samra *et al.* 1996) and nucleic acid analysis (Simon *et al.* 1993a; Wyss & Bonfante 1993; Bonito *et al.* 1995; Lanfranco *et al.* 1995; Sanders *et al.* 1995; Mello *et al.* 1996; Rosendahl & Taylor 1997; Zézé *et al.* 1996, 1997; Antoniolli *et al.* 2000). With the detection of AM-specific genes eg. Gimchs1 and Gimchs3 (Lanfranco *et al.* 1999; Delp *et al.* 2000), and glomalean specific primers eg. Glomales-specific primer VANS1 and Gigasporaceae-specific primer VAGIGA , specific molecular techniques eg. nested PCR, competitive PCR and in situ PCR were used to study the variations among the AM fungi thereby signalling an advance in studying genetic processes in AM fungi (Bago *et al.* 1998; Vandenkoornhuyse & Leyval 1998; Pringle *et al.* 2000; Jacquot *et al.* 2000). Quantitative analysis of *Glomus mosseae* in the roots of host species using competitive PCR (Edwards *et al.* 1997) and for *Gigaspora* and *Scutellospora* spp. by quantitative analysis of amplified 18S rRNA from onion roots was successfully reported, which was found to be correlated with the colonisation percentage (Oba *et al.* 2002).

However, doubts were raised for the specificity of the proposed AM-specific primers (Clapp *et al.* 1999; Schüssler *et al.* 2001). Furthermore, most of these methods are still in their developmental stages and in most cases, may be applicable to only a few species, rather than all the members of the population. Currently, none of these methods can be used reliably to quantify AM fungal biomass in the soil.

1.1.4 Populations of Mycorrhizal Fungi in Natural Ecosystems

AM fungi can be found in most terrestrial ecosystems, including temperate and tropical grasslands, desert ecosystems and tropical forests (Read 1991). The distribution of species appears to be global; at least one species (*Glomus intraradices*) has been reported from almost every continent (Morton 1990). Amongst natural ecosystems, the diversity of AM fungi in sand dunes has been extensively studied using the spore-based methods. In general, stabilised sand dunes have greater species diversity than disturbed dunes (Koske 1975; Giovannetti & Nicholson 1983). In a natural tall grass prairie, Bentivenga & Hetrick (1991) found the spores of14 different AM species with *Glomus ambisporum* being the dominant species present.

AM species diversity in different man-managed ecosystems has been found to range from 2-25 species in different studies. Species diversity was low in arid

grasslands, pastures and crops eg. wheat fields (Molina *et al.* 1978; Abbott & Robson 1982a; Daniels Hetrick & Bloom 1983), and high in agro-forestry sites, corn-soybean rotation fields and mown pastures (Walker *et al.* 1982; Kurle & Pfleger 1996; Bever *et al.* 1996). Boerner *et al.* (1996) also reported that mycorrhizal infectivity was lower in an agricultural field than a mature forest.

AM species diversity may be adversely affected when natural ecosystems are disturbed, such as their clearing for agricultural use or mining (Johnson *et al.* 1992a). Some agricultural practices eg. pesticide application, tillage or particular cropping sequences may also affect species diversity (Jasper, *et al.* 1987, 1989; Johnson *et al.* 1991, 1992). The effect of these changes in AM species diversity on functional aspects of the symbiosis is unclear. For example it is not known whether the dominant fungal species in a mixed population is also the one that is the most infective or the most beneficial to the plant (Barea & Jefferies 1995).

1.2 Mycorrhizal Infectivity

Mycorrhizal infectivity (or AM infectivity) is defined as the ability of a mycorrhizal fungus to colonise the roots of a host plant. It is influenced by fungal characteristics and by the occurrence and density of mycorrhizal propagules in the soil or in an inoculum. Different AM species show differences in the rate at which they colonise plant roots (Gianinazzi & Gianinazzi-Pearson 1986; Sieverding 1991). This may be due to differences in the total AM fungal biomass present as an inoculum, or to differences in the infective potential of the AM fungal propagules (eg. spores or hyphal containing root fragments) that constitute the inoculum (Abbott & Robson 1981a, b; Wilson 1984; Daniels *et al.* 1985). Consequently, it is important to be able to determine the density of different AM fungal propagules in an inoculum sample in order to establish its infectivity. However, it is also difficult to determine the relative contribution of the different propagules in an inoculum to AM infectivity. For example, % root colonisation, which is one of the most important measures of infectivity, may be influenced not only by various propagule factors eg. the number, dormancy and rate of germination of spores, but also by the capacity of the host root system to become mycorrhizal (Smith & Read 1997). Propagule generation also seems to be a factor influencing AM infectivity. A decrease in the infectivity of propagules (infected root pieces and spores) was noted by Plenchette *et al.* (1996)

over three successive generations. Thus despite the importance of the propagules in AM infectivity, there needs to be more work done to establish the relative efficiencies of different AM propagules in this process.

Some soil factors eg. periodic wetting and drying, may also affect the rate of mycorrhizal colonisation of plant roots (Pattinson & McGee 1997). The effect of cropping sequence on mycorrhizal colonisation, spore production and AM infectivity has been studied (Black & Tinker 1979; Dodd *et al.* 1990; Johnson *et al.* 1992), but there is still relatively little is known about specific plant-fungus interactions and their effect on AM infectivity of soil.

1.2.1 Components of AM Infectivity

1.2.1.1 Spores

Spores are the important propagules of AM fungi and are considered to be more resistant to adverse soil conditions than infected root fragments or hyphae (Abbott & Robson 1982b). Spores are produced either singly on the extraradical mycelium or aggregated into well-defined structures called sporocarps. The spores of AM fungi range in size from about 50 to 600 μm in diameter and are some of the largest known fungal spores in nature (Mosse 1981). Their basic function is to preserve genetic information and enable survival of AM fungal species during unfavourable periods.

Although spores are considered to be the most important propagule in terms of AM infectivity (Chuang & Ko 1981), the density of spores in an inoculum is not always correlated with % root colonisation (Smith & Read 1997). In fact Louis & Lim (1987) observed an inverse correlation, where increasing spore density was associated with a decrease in root colonisation. Adelman & Morton (1986) and Baon *et al.* (1992) also observed more spores in a sample than could be estimated by a most probable number (MPN) method, suggesting that probably not all spores isolated from soil are viable. Often, no correlation is observed between the number of spores in a soil sample counted with the aid of a microscope and the number of spores estimated by MPN methods (Powell 1977).

Spores as propagules offer the potential of generating uncontaminated single spore isolate cultures. Monoaxinically-produced spores produced essentially the same amount of colonisation as root segments (Vimard *et al.* 1999). However, when

spores are collected from field soils they are generally found to be incapable of starting trap cultures (Brundrett *et al.* 1999).

1.2.1.2 Extra-radical Hyphae

As mentioned earlier, mycorrhizal roots produce large amounts of external (extra-radical) hyphae. This hyphal network is well known for its capacity to link different plants together (Harley 1989; Graves *et al.* 1997; Schreiner *et al.* 1997), and for its capacity to exchange nuclear and protoplasmic contents within the hyphae originating from different spores of the same isolate through anastomosis formation (Giovannetti *et al.* 1999). In undisturbed soil the hyphal network makes a very important contribution to AM infectivity. Read *et al.* (1985) were the first to emphasize the importance of the living hyphal network in initiating rapid colonisation in seedlings.

The survival of the hyphal network in soil has been studied and is found to retain infectivity even when the host plant is dead or dormant. The hyphal network was found to survive in the hot and dry soils of Australia (McGee 1989; Jasper *et al.* 1989, 1993; Braunberger *et al.* 1994) and in the very cold soils of Canada (Addy *et al.* 1994). After a period of dormancy the AM hyphae are able colonise living roots when the growing season returns.

Disturbance of the mycorrhizal hyphal network may result in reduced AM infectivity of the soil (Birch 1986; Jasper *et al.* 1989; Jasper *et al.* 1991, 1992; McGonigle *et al.* 1990a; Merryweather & Fitter 1998). In a study by Roldan *et al.* (1997), AM infectivity in the soil declined rapidly during 5 years of agriculture, but it recovered to its original infectivity over the next 45 years if left abandoned. In a study by Hutton *et al.* (1997), the removal of host plants and disturbance of the AM hyphal network due to bauxite mining was considered to be the main factor contributing to a decline in AM infectivity which was re-established slowly over 12 years.

AM hyphal density in the soil has been mainly studied in relation to nutrient uptake and soil stabilisation, with little emphasis on infectivity. Hyphal density is affected by soil temperature with some AM species eg. *Glomus* spp more abundant in warm soils (Bhaskaran & Selvaraj 1997; Braunberger *et al.* 1997) while others eg. *Acaulospora* spp and *Scutellospora* spp, are more abundant in cold soils (Braunberger *et al.* 1997). The total length of extraradical mycelium in the soil has

been measured in several studies (Bethlenfalvay 1982a,b; Schubert *et al.* 1987). Miller *et al.* (1995) measured a peak in total hyphal length of 111 m cm^{-3} in a tall-grass prairie soil and 8 m cm^{-3} in a cool-season pasture soil. Although the biomass of AM fungal hyphae increases in roots during plant growth, it decreases in soil (Zhao *et al.* 1997).

With respect to infectivity, it is more important to study the contribution of active mycelium to % root colonisation or plant growth than to measure the total biomass of hyphae in the soil. Sylvia (1988) found a significant correlation between active hyphal length and root colonisation, while Miller *et al.* (1995) suggested that root morphology (length and mass) could have a strong association with the hyphal length. Hyphal activity was successfully determined by measuring the concentration of glomalin on strips of horticultural film using the ELISA and Bradford protein assay (Wright & Upadhyaya 1999). Specific phospholipid fatty acids have also been used to estimate the biomass of AM fungal hyphae in the soil (Olsson *et al.* 1995, 1998 1999).

Another problem associated with assessing the biomass of AM fungal hyphae in the soil is that AM hyphae cannot readily be distinguished from the hyphae of other fungi in the soil. Some researchers have attempted to get around this problem by assuming that the biomass of non-mycorrhizal fungal hyphae in the soil will be the same in control (non-mycorrhizal) and experimental (mycorrhizal) soil. Hence the biomass of AM fungal hyphae in experimental pots of soil can be determined by subtracting the amount of fungal hyphae present in non-mycorrhizal control pots of soil (Abbott & Robson 1985; Sukarno *et al.* 1996). However, this procedure requires verification using other methods for estimating the amount of AM hyphae present. Attempts have been made to distinguish AM fungal hyphae from hyphae of saprophytic fungi using specific fatty acid methyl esters (Stahl & Klug 1994; Müller *et al.* 1994; Larsen *et al.* 1998; Olsson *et al.* 1999; Jansa *et al.* 1999). This has been successful in controlled pot experiments where the chances of contamination of the soil by other fungi are reduced (Olsson *et al.* 1999).

1.2.1.3 Infected Root Fragments

Root fragments are important propagules of AM fungi (Hall 1976; Powell 1976; Williams 1990) and depending upon their availability and affectivity will have a

direct effect on the AM infectivity of the soil. However, the capacity for AM fungi to survive in root fragments is largely unknown, although in one experiment the hyphae remained effective in dead root fragments for up to 6 months (Tommerup & Abbott 1981). In another case the infectivity of root fragments and spores was found to decline during a two year period of fallow (no plants) (Thompson 1987). In pot experiments, it was noted that the distribution of root fragments was important in influencing the rate of colonisation in *Trifolium subterraneum* seedlings (Smith & Smith 1981).

1.2.2 Factors Influencing AM Infectivity

1.2.2.1 Spore Germination

The ability to germinate, the pattern of germination and the quantity of mycelium produced are characteristics of spore germination which show a high degree of variation within and between species (Hepper & Smith 1976; Giovannetti & Gianinazzi-Pearson 1994) and hence are used as taxonomic characters (Walker & Sanders 1986). These spore characteristics may have a major effect of the AM infectivity of the soil.

Spore germination may be influenced by many factors (Table 1.1) including edaphic factors such as temperature, pH, moisture, texture, presence of heavy metals and soil nutrient levels. (Daniels Hetrick 1984; Hepper 1984; Frey & Ellis 1997). Variation in these factors can affect the types of AM fungi present in different soils (Sieverding 1991). For example, nutrient-rich high P soil compost with chicken litter was found to enhance spore production by *Glomus* spp. (Douds *et al.* 1997).

Table 1.1. Effect of various factors on germination of AM fungal spores

Factor	Condition	Effect on spore germination
Soil temperature	Low	Rapid germination (Hepper & Smith 1976).
Salt Concentration of soil	High	Reduced germination (Juniper & Abbott 1993),
P Concentration of soil	High (16 mM)	Inhibition of spore germination (Tawaraya *et al.* 1996)
Heavy Metals (eg. Zn, Mn, Cd)	High	Reduced germination (Galli *et al.* 1994)
Organic Acids & Sugars	High	Reduced germination (Smith & Read 1997)
Spore Dormancy	Present	Negative effect (Tommerup 1983; Giovannetti & Citernesi 1993)
Soil Fungi	Present in soil	Enhanced germination (Azcon-Aguillar *et al.* 1986)
Soil Bacteria	Presence or Absence	Some promote some inhibit (Fitter & Garbaye 1994)
Bacterial Cell-free Extracts	Presence	Enhanced germination (Tilak *et al.* 1990)
Fungicide Application (eg. Benomyl, Pentachloronitrobenzene)	20 mg/kg^{-1} soil	Inhibit ion of spore germination (Schreiner & Bethlenfalvay 1997)
Surface Sterilisation	Spores	Negative effect (Wilson *et al.* 1989).

1.2.2.2 Root Colonisation

As with spore germination there are many factors that have been shown to affect the rate and extent of root colonisation by AM fungi. Some of these factors are listed in Table 1.2.

Table 1.2. Effect of various factors on root colonisation by AM fungi.

Factor	**Condition**	**Effect on Colonisation**
Propagule density	Higher	Positive effect (Carling *et al.* 1979; Smith & Walker 1981; Wilson 1984)
Soil temperature	Higher	Positive effect (Bhaskaran & Selvaraj 1997)
Soil nutrients	Deficient in Ca	Reduction in colonisation (Habte & Soedarjo 1995).
Soil moisture	Flooding or wetting	Reduction in colonisation (Mosse 1981; Miller 2000)
Soil compaction	Higher	Negative effect (Entry *et al.* 1996)
Root exudates from host plants	Plants in P-deficient soil	Increased colonisation (Tawaraya *et al.* 1998)

1.3 Estimation of the Biomass of AM Fungi

Quantification of biomass or number of AM fungal propagules in soil is an important pre-requisite to estimating the AM infectivity of soil. Theoretically, infectivity may be directly proportional to the existing fungal biomass and number of spores present in soil at any given time. However, this is not the case in many instances because the fungal propagules are either inactive and therefore cannot effect colonisation, or are dormant for varying lengths of time. Hence, measurement of fungal biomass or number of spores can only provide an estimate of AM infectivity in the soil. Therefore, we require methods, which cannot only measure the total biomass of propagules in the soil but can also differentiate between viable and non-viable propagules. Currently, due to the lack of existing good measures and the fact that mycorrhizas must be studied along with the host plant due to their obligate nature, it is extremely difficult to estimate the exact biomass of AM fungi in the soil.

There are both direct and indirect methods for estimating the number or biomass of microorganisms in the soil (Herbert 1990). The direct methods can be separated into total count methods, ie those which attempt to count a total number of selected organisms in a given sample irrespective of whether they are viable (living) or non-viable (dead), and viable count methods, which differentiate between living and dead organisms or propagules by assessing their ability to grow. Indirect methods involve the estimation of a specific component of a microbial cell, which is present in direct proportion to the amount of cell biomass eg. estimation of ATP, chitin or polysaccharides in bacteria; phospholipids in bacteria and fungi; photosynthetic pigments in phytoplanktonic algae, and DNA in all types of microorganisms.

Some commonly used methods of estimating the number and biomass of AM fungal propagules are discussed below.

1.3.1 Conventional Microscopic Techniques

Conventionally AM fungal populations in soil and plant roots have been quantified using microscopic methods. Quantitative measurement of intra-radical hyphae inside the root cells is achieved either by morphometric methods (Phillips & Hayman 1970; Kormanik *et al.* 1980; Brundrett *et al.* 1984) or by image analysis of stained root sections (Smith & Dickson 1991). Giovannetti & Mosse (1980) have evaluated these

methods, and Trouvelot *et al.* (1982) and McGonigle *et al.* (1990b) have suggested a number of new methods. In soil, extra-radical mycelium of AM fungi has been measured by different wet sieving techniques combined with microscopical quantification of the mycelium. The details of these methods will be described in the following paragraphs.

However, apart from vital staining, these methods do not differentiate between dead and live fractions of the AM fungal biomass. Also, these methods do not allow for any systematic or functional separation of different fungal mycelia (Sylvia 1992). Moreover these methods are generally not reliable in field soils, and those methods, which are spore-based, cannot be applied to those AM fungi, which do not produce spores.

1.3.1.1 % Root Colonisation

Estimation of infected root length forms the basis of many methods of quantifying AM fungal biomass, because of the need to use trap plants to detect the presence of infective propagules of the obligate AM fungi. The AM fungal hyphae, which colonise inside the roots, can be visualised by a combination of clearing the root cellular contents with KOH (Gardner 1975) and staining the AM fungal hyphae with trypan blue or chlorazole black E prepared in lactoglycerol (Bevege 1968; Phillips & Hayman 1970; Brundrett *et al.* 1984).

The total length of plant roots colonised by AM fungi is then estimated, most commonly, by using the grid line intersect method (Newman 1966; Tennant 1975; Giovannetti & Mosse 1980). According to the recommendations of Giovannetti & Mosse (1980), a minimum of 100 intersections should be used to assess a sample and accuracy is improved if samples are re-randomised and counted several times. In order to assess the different components of the mycorrhiza eg. size of arbuscules and vesicles and length of internal hyphae, a compound microscope with an eye-piece cross-hair is used which is moved to randomly selected positions (McGonigle *et al.* 1990b).

There is a need for many safety precautions to be observed due to the toxic nature of the stains and chemicals used in this procedure. KOH and phenol, used in stains, can be highly toxic and stains eg. trypan blue and chlorazole E are suspected to be carcinogenic (Coombes & Haveland-Smith 1982). Moreover, the performance

of the stains can be plant specific. Gange *et al.* (1999) found that estimations of % root colonisation differed significantly with different stains for a number of plant species. Modifications have been suggested with the stains used - eg. the use of methyl blue or aniline blue has been suggested by Grace & Stribley (1991), and acid fuschin can be used in combination with fluorescence microscopy to stain AM fungal structure inside roots (Merryweather & Fitter 1991).

Alternatively, the presence of AM fungi inside roots can be confirmed using vital stains that measure the fungal activity eg. succinate dehydrogenase activity (Schaffer & Peterson 1993; Tisserant *et al.* 1993). However, this gives the same information that is provided by measures of % root colonisation.

1.3.1.2 Quantification of External Mycelium

External hyphae form a substantial part of any mycorrhiza and some traditional techniques are used to measure the biomass of external hyphae. The most commonly used direct method is to measure the length of hyphae in the soil, convert this measurement into volume and then into biomass (Bååth & Söderström 1979; Bakken & Olsen 1983). Hyphal length is measured using a grid-line intersect method (Abbott *et al.* 1984; Sylvia 1986; Miller *et al.* 1987). However, a major problem associated with this method is the difficulty of distinguishing AM fungal hyphae from the hyphae of other fungi. Some researchers have suggested it may be possible to do this because the diameter of the hyphae of AM fungi may be greater than that of other fungi (Graham *et al.* 1982; Bethlenfalvay & Ames 1987). However, others disagree with this (eg. Abbott & Robson 1985).

Sylvia (1992) listed a number of direct and indirect methods for quantification of external mycelium. The indirect methods include estimating the % root colonisation of a receiver plant (Warner & Mosse 1983; Schüepp *et al.* 1987; Miller *et al.* 1989) and estimating the amount of soil adhering to roots of the host plant, after shaking it vigorously. With the latter method, the amount of soil adhering to the roots is correlated with other estimates of external hyphae and also with the plant growth response (Graham *et al.* 1982). However, Kough & Linderman (1986) criticised this approach because of the many factors that may affect the quantity of soil attached to roots.

The measurement of chitin has been used to estimate the biomass of AM hyphae in soil (Hepper 1977; Vignon *et al.* 1986; Bethlenfalvay & Ames 1987). But as chitin is also present in other microorganisms and the exoskeleton of insects, the validity of this method has been questioned (Sharma *et al.* 1977; Jarstfer & Miller 1985). Some vital stains are also used to estimate the biomass of living fungal hyphae in soil eg. fluorescein diacetate (FDA), which is acted upon by hydrolytic enzymes to release fluorescein (Söderström 1977; Domsch *et al.* 1979; Schnurer *et al.* 1985, 1986).

Most of the techniques described above do not differentiate between living or dead fungal hyphae. Whilst there are numerous methods available to test the viability of fungal hyphae such as the reduction of iodonitratetrazolium (Sylvia 1988), it must be concluded that at present, there is no single method based on conventional microscopy, that can provide an unambiguous estimate of the amount of external hyphae of AM fungi in the soil.

1.3.1.3 Spore Counting

There are several methods for separating spores from soil (Gerdemann & Nicolson 1963; Daniels & Skipper 1982; Tommerup 1992). With most methods, the spores are separated from the soil by passing a known volume of soil through a series of sieves of different pore size and then fractionated according to their size, using sucrose density gradient. The spores are then manually counted under microscope.

Some alternative methods, which do not involve sieving the soil, have been developed. These include applying a stream air, of known velocity, upward through a bed of dry soil. The AM fungal spores and other propagules eg. viable hyphae in dead roots are sorted according to their sedimentation velocity (Tommerup 1982; Tommerup & Carter 1982). This method is useful for sandy soils but it can be inefficient for highly aggregated soils. Another method, which uses a nematode-counting slide (Daniels & Skipper 1982). This is useful for heavier soils with high spore densities and involves the direct counting of spores in a soil-water suspension. If the number of spores in a soil sample is expected to be above 10 g^{-1} soil and the amount of soil to be sampled is small, then a plate method can be used (Smith & Skipper 1979). This method involves suspending the soil sample in a known volume

of water, shaking it vigorously and pipetting the supernatant onto a filter paper in a Buchner funnel. The separated spores can then be counted. In heavy clay soils it may be useful to precipitate the clay particles out of the water suspension before applying it to the filter (Pacioni 1992).

There are several limitations with measuring spore number as a parameter of AM infectivity in soil (Smith & Read 1997). In particular, not all the spores present in a sample may be viable - some may be dormant (Tommerup 1992), and some may have been attacked by soil microorganisms (Lee & Koske 1994). The viability of spores can be checked using various bioassays, but performing these bioassays can be a lengthy and tedious process.

1.3.1.4 Most Probable Number (MPN) Method

The most probable number (MPN) method, also known as the dilution method, permits the estimation of the size of a microbial population without actually counting single cells or spores. It works on the principle that AM population can be recognised in terms of enhancement of growth of a host plant.

The MPN method was first used to estimate propagules of AM fungi by Maloy & Alexander (1968) and has been since used by many researchers (Alexander 1965; Porter 1979; Wilson & Trinick 1982; McGraw & Hendrix 1986). The method is especially useful for assessing the number of viable propagules in a sample and is particularly useful if the propagules are difficult to isolate or their form is not known. Porter (1979) observed that the MPN method gave a more realistic estimate of the number of infective propagules of AM fungi in soil, than just counting of spores. Similar observations were made by An *et al.* (1990), who found that the MPN method detects all AM propagules capable of infecting the host plant, including those embedded in soil organic matter. Also, by using the appropriate bioassay host plant minor components AM fungal community may be detected (McGraw & Hendrix 1984; An *et al.* 1990).

Like any other bioassay, the MPN method has been criticised, mainly on the basis of sensitivity of the host plant used. Plant growth conditions (eg. temperature, duration) will vary in different MPN experiments, and may give different results with the same soil sample (Wilson & Trinick 1982). Also the specificity of the individual AM fungi in the test soil for the bioassay host may vary (Adelman & Morton 1985)

and the MPN test fails to take into the dormancy of spores in some AM fungi (Tommerup 1983). However, the most positive aspect of MPN method is that it relates well to the AM infectivity of the soil.

1.3.2 Biochemical and Molecular Methods of Detection and Quantification of AM Fungi

1.3.2.1 Isoenzymes

Isoenzymes are defined as multiple molecular forms of an enzyme having similar enzymatic properties. Isoenzyme analysis involves the separation of enzyme proteins by various electrophoretic techniques such as starch gel electrophoresis, pulse gel electrophoresis or electrofocussing. The underlying principle is that the isoenzymes, which are coded by different alleles or separate genetic loci, possess different electrophoretic mobility. The banding pattern of the isoenzymes thus reflects differences in the DNA sequence coding for the isozymes. Consequently, the technique has been found to be more useful for species differentiation within AM fungi than for quantifying AM infection of plant roots.

Electrophoretic analysis of isoenzymes was first used for the identification of an ectomycorrhiza formed by *Pisolithus* on *Eucalyptus* (Seviour & Chilvers 1972). This was followed by a similar study on AM fungi by Gianinazzi-Pearson & Gianinazzi (1976). Since then there have been several reports where the identity of an AM fungal isolate has been confirmed using isoenzyme markers. For example, isozyme markers were used to show that different geographic isolates of *Glomus clarum* belonged to the one species. (Sen & Hepper 1986). However, this differentiation was confined to the species level and differences within species were not detected. In another study, Hepper *et al.* (1988) assessed the electrophoretic mobility of isozymes of six enzymes active in the resting spores of a large number of AM fungi. The resultant banding patterns were used to compare and cluster the AM species based on similarities. Shankar & Varma (1993) also found isoenzyme analysis of AM fungal spores to be useful in determining taxonomic relationships between AM fungi. Most recently, Timonen *et al.* (1997) have attempted to characterise the host genotype and study diversity in Scots pine ectomycorrhizas using isoenzyme analysis combined with PCR-RFLP analysis.

Although used mainly for the identification of AM fungal species, attempts have been made to quantify AM fungal colonisation of plant roots by relating the degree of colonisation to a visual assessment of fungal isoenzyme activities (Hepper *et al.* 1988; Rosendahl *et al.* 1989).

Isoenzyme analysis is limited in many ways. It relies on gene expression for the presence of a particular isoenzyme. Gene expression may be affected by environmental, cultural or internal factors. The analysis may also be affected by the formation of artefact bands of secondary isoenzymes, which can arise from the proteolysis of the sample during extraction and storage of proteins (Harris & Hopkinson 1976). Host background activity has also been observed in some cases (Rosendahl & Sen 1992). However, one of the biggest criticisms of using isoenzymes for identification or quantification of AM fungi is that the enzymes are only detectable in metabolically active mycelium. Also, it is difficult to be certain that other microorganisms, colonising the roots eg. bacteria, do not cause the isoenzyme activity detected.

1.3.2.2.Antibodies

Another approach using proteins for the detection of AM fungi involves the development and use of antibodies specific to antigens of the fungal species. The antibodies produced can be either monoclonal ie. specific for particular antigens in specific organisms, or polyclonal which are usually non-specific and cross react with other antigens. Both polyclonal (Aldwell & Hall 1986; Kough & Linderman 1986) and monoclonal antibodies (Halk & DeBoer 1985; Morton 1987) have been used successfully for quantification of AM fungal hyphae in soil and plant root samples.

Glomalin is another glycoprotein, which is used in the detection of AM fungi in soil. Glomalin is produced by hyphae of AM fungi and plays an important role in soil organic matter particularly in binding water-stable soil aggregates (Rillig *et al.* 2002). The extraction and measurement of glomalin is achieved following extraction by a pressure cooker technique (see Wright & Jawson 20001) or by an indirect immunofluorescence assay by which it could be detected on hyphae or roots and on surface of the soil aggregates (Wright 2000). Glomalin appears to persist for quite

long time in soil, so that its precise value in measurement of AM hyphae in soil is not clear.

1.3.2.3 DNA Techniques

There are many examples of studies using DNA techniques for detection, identification and population studies of AM fungi (Simon *et al.* 1992, Wyss & Bonfante 1993; Sanders *et al.* 1995; Zézé *et al.* 1996; Bonfante 1997; Lanfranco *et al.* 1999; Antoniolli *et al.* 2000; Oba *et al.* 2002).

The polymerase chain reaction (PCR) technique was first used with AM fungi by Simon *et al.* (1992a). They amplified and sequenced the 18S rRNA gene using universal primers NS1, NS2, SS38 and NS21 using DNA from a small number of spores of selected AM species. The sequences were different for different AM fungal species. Simon *et al.* (1992b) went a step further and designed a Glomales specific primer (VANS1). Using this primer it was possible to amplify and sequence the 18S rRNA gene from several different species of AM fungi representing all the genera in the Glomales. Simon *et al.* (1993b) also suggested that this technique could be used to classify AM fungi in a manner consistent with that based on spore morphology (Morton 1990). Sanders *et al.* (1995) used universal primers ITS1 and ITS4 to amplify and sequence the highly conserved 5.8S rRNA and revealed considerable differences between different genera in the Glomales. These studies also demonstrated that amplification of specific DNA segments from the small subunit of fungal rDNA can be used to identify AM fungi in plant roots.

Further studies have been targeted towards obtaining DNA probes with specificity towards different species within genera of the Glomales and differentiation among isolates of a species, which was still lacking. Research done by Clapp *et al.* (1995) resulted in the design of primers (VAGIGA, VAGLO and VAACAU) with taxonomic specificity at the genus level, and which were subsequently used by Sulistyowati (1995) to detect different genera of AM fungi in colonised roots. At the same time Bonito *et al.* (1995) used the primer pair VANS1 and NS2 to detect *Glomus intraradices* in the roots of different host plants.

Lanfranco *et al.* (1995) used RAPD-PCR to generate a specific primer PO-M3, which was used to differentiate some isolates of *Glomus mosseae*. Zézé *et al.* (1996) successfully isolated and characterised a highly repetitive sequence from the

genome of *Scutellospora castanea*, which could be used to amplify this sequence from spores and plant roots colonised by this AM species. This probe was specific for *S. castanea* and did not amplify the DNA of other AM species. Use of RAPD-PCR (using M13 minisatellite sequences as primers) showed a similar pattern for spores of *Gigaspora margarita*, obtained either *in vitro* or *in situ* (Gadkar *et al.* 1997). However, the RAPD-PCR pattern obtained for the spores of *G. margarita* and *G. gigantea*, were different suggesting that minisatellite sequences could be exploited for identifying and differentiating AM fungi.

Further advances in molecular techniques included the identification of DNA sequences from smaller subunit (SSU) of ribosomes, which were helpful to detect, describe and quantify AM fungal species. Vandenkoornhuyse & Leyval (1998) suggested that SSU rDNA sequences could be used in description of Glomales and Chelius & Triplett (1999) used PCR amplification of SSU rDNA gene to detect the presence of *Glomus intraradices* in soil and roots of turfgrass (*Agrostis palustris*). Bago *et al.* (1998) went a step further in successfully amplifying, in-situ, the SSU rDNA sequences using PCR with fluorescently labelled Glomalean-specific primers and then directly detecting the amplified sequences by epifluorescence microscopy. This modified, in-situ amplification presented the possibility of studying transient and localised genetic processes of AM fungi during their development in the roots. Van Tuinen *et al.* (1998) found nested PCR to be highly sensitive technique in characterisation of root colonisation profiles, a conclusion which was later supported by Jacquote *et al.* (2000), who also recommended that the variability of D1 and D2 domains of larger subunit could be used to design primers for the purpose of differentiating AM fungi at species level.

Quantification of AM fungi has also been attempted inside the host roots by using competitive PCR for example, for *Glomus mosseae* (Edwards *et al.* 1997). Oba *et al.* (2002) successfully used polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP), not only to quantify the fungal biomass of two AM fungi (*Gigaspora margarita* and *Scutellospora cerradensis*) but also to differentiate the two. They used AM-specific primers VANS1 to amplify 18S rRNA fungal gene in the roots and digested the amplified product with HincII restriction enzyme. Identification of genes, which could be linked to the

development of AM fungi inside the roots, was another line of research followed by various researchers in recent attempts. Thus, Lanfranco *et al.* (1999) identified 3 chitin synthetase genes (Gimchs1, Gimchs2, and Gimchs3) using reverse transcription polymerase chain reaction (RT-PCR) which were expressed during root colonisation but were not revealed during spore germination. Similarly, Delp *et al.* (2000) used differential display PCR to identify three genes that were differentially expressed in symbiosis *by Glomus intraradices* and barley roots. Martin-Laurent *et al.* (2001) identified Bam HI element in psam3 gene, which showed enhanced expression during the early stages of development of AM colonisation in the roots of *Pisum sativum* L.

These techniques have demonstrated considerable potential for the detection, identification and quantification of AM fungi within plant roots. However, as yet they have not been applied extensively to the study of AM fungal populations. Some practical difficulties associated with the DNA methods include the genetic variability inherent in the multinucleate status of AM fungal spores, problems with not having enough fungi in the plant roots for PCR amplification, and expensive DNA kits. The other more serious problem with molecular techniques is the detection of non-specificity of presumably AM-specific primers. During a study of the amplification of SSU rRNA sequences using Glomales-specific primer VANS1 and Gigasporaceae-specific primer VAGIGA in the roots of bluebell, Clapp *et al.* (1999) found that primer VANS1 was not so specific and in addition to Glomalean (*Scutellospora*) sequences, a number of different sequences from Ascomycetes were also obtained. In a study of genetic diversity at three levels – within isolate at spore level, between the individuals of a single soil sample and between the individuals of different soil samples using amplification of rDNA of ITS region, Pringle *et al.* (2000) reported a very complex pattern of rDNA within Glomales. In this study, more genetic variation was reported in the ribosomal sequences from spores of the same species than from different species of Glomalean fungi taken from different soils, challenging the use of genetic methods in identification of AM fungi. In another extensive study of testing the specificity of purported AM-specific primers using 51 partial Glomales SSU rRNA gene sequences from 33 Glomales isolates, Schüssler *et al.* (2001) found that most of the published primers, including VANS1,

were not specific for the Glomales. VANS1 primer found homology with only less than half of the AM fungal sequences investigated by Shüssler *et al.* (2001). These recent contradictory findings have indicated the limitations of the use of molecular techniques in studying AM fungi on a large scale.

1.3.3 Detection and Quantification of AM Fungi Using Fatty Acid Analysis

Fatty acid analysis may be able to overcome some of the drawbacks experienced with other methods for the detection and quantification of AM fungi. Recent studies have demonstrated that differences in microbial community structure and biomass, can be detected by analysis of the fatty acid methyl ester (FAME) profiles derived from lipids extracted from the soil (reviewed by Zelles 1999). The fatty acid profiles may be derived from both cellular (living) and non-cellular lipids on the soil (referred to as FAME analysis), or from specific lipid classes such as phospholipids or neutral lipids fractions of the soil. Phospholipids are a component of the membranes of all living cells (Kates 1964). They breakdown rapidly in the soil following cell death (White *et al.* 1979). Analysis of phospholipid fatty acids (PLFAs) has been used to characterise and quantify components of the viable microbial biomass in the soil (Frostegard & Bååth 1996). Neutral lipids occur in the storage structures of microorganisms eg. the spores and vesicles of different fungi including AM fungi (Cooper & Lösel 1978; Nagy *et al.* 1980; Jabaji-Hare *et al.* 1984;Olsson *et al.* 1999).

The amount and types of fatty acids vary in different groups of organisms and different organisms may have different combinations of these fatty acids. This information can be used for identification and differentiation between organisms. Since fatty acids can be volatilised following methylation, they can be readily analysed by gas chromatography (Moss *et al.* 1980; Moss 1981; Vestal & White 1989; Zelles & Bai 1993; Cavigelli *et al.* 1995).

FAME analysis has been developed as an identification system for bacteria and a comprehensive database containing the FAME profiles of most culturable bacterial species is available (Sasser 1990). A similar but less comprehensive database is available for fungi (Johnk & Jones 1993; Stahl & Klug 1996; Jansa *et al.* 1999).

FAME and PLFA analysis has been used widely to characterise the composition of soil microbial communities (Haack *et al.* 1994; Cavigelli *et al.* 1995;

Bardgett *et al.* 1997; Zelles 1997), and for monitoring changes in the composition of soil microbial communities (Bååth *et al.* 1992; Frostegard 1995; Zelles 1999). Other applications include the use of selected fatty acids as biomarkers for bacteria and fungi and to calculate the fungal/bacterial ratio for a soil community (Frostegard & Bååth 1996; Zelles 1999). Several studies have used this fatty acid based fungal/bacterial ratio to follow seasonal variations in soil microbial communities (Rajendran *et al.* 1992; Frostegard *et al.* 1993; Frostegard & Bååth 1996; Bardgett *et al.* 1997; Riechardt *et al.* 1997; Frostegard *et al.* 1997).

Using fatty acid analysis for detection and biomass estimation of AM fungi in soil and plant roots was possible because lipid reserves in spores of AM fungi are generally high (about 45% on a dry weight basis) (Beilby 1980; Beilby & Kidby 1980), and in some cases up to 80% (Olsson, PA, pers. com.). Also whilst the broad types of lipid found in AM fungi are not different from other fungi, there are sufficient differences in the fatty acid composition of the lipids of AM fungi to differentiate them from most other fungi. Fatty acids common in the lipids from different AM fungal structures but uncommon in other fungi include 16:1ω5c, 18:1ω7c, 18:3, 20:3, 20:4 and 20:5 (Olsson *et al.* 1995). These fatty acids have also been detected either exclusively or in higher amounts in plant roots colonised by AM fungi compared to uncolonised roots (Beilby 1980; Lechevaliar & Lechevaliar 1988; Pacovsky & Fuller 1988; Müller *et al.* 1994; Olsson *et al.* 1995).

The second most important finding after discovering the association of fatty acids with AM fungi, was establishing the fact that fatty acid compositions and FAME and PLFA profiles of AM fungi were stable, specific to organisms and heritable (Bentivenga & Morton 1994; Bentivenga & Morton 1994a; Graham *et al.* 1995). FAME and PLFA profiles have been found to be stable when the cultures are stored at 4.5°C (Peterson & Klug 1994) and constant as the cultures age (Olsson & Johansen 2000). Furthermore, Bentivenga and Morton (1996) also found that the FAME profiles were in congruence with the morphological characters in the family Gigasporaceae using phylogenetic study. This information lead fatty acid analysis to be developed as an important technique in quantifying AM fungal colonisation in roots and physiological cost of the fungus in roots (Peng *et al.* 1993; Graham *et al.* 1996). Olsson *et al.* (1995) developed and applied PLFA and NLFA (neutral lipid

fatty acids) techniques for quantification of AM fungi in the form of hyphae and spores inside and outside the roots to estimate internal and external fungal biomass. Several studies since then have shown that fatty acid 16:1ω5c is correlated with the level of root colonisation by AM fungi (Graham *et al.* 1995; Johansen *et al.* 1996; Olsson *et al.* 1995, 1998, 1999). Thus it was established that there was potential for this fatty acid to be developed as a specific biomarker for AM fungi. Notably, this fatty acid was commonly reported from *Glomus* spp. (Jabaji-Hare 1988, Graham *et al.* 1995; Olsson *et al.* 1995; Jansa *et al.* 1999). Fatty acid 16:1ω5c is also reported in a few other fungi eg. *Mucor* spp. (Jansa *et al.* 1999; Olsson 1999) and some bacteria eg. *Flexibacteria* (Nichols *et al.* 1986), but the amounts are generally very low (<5% of total lipids present), which does not restrict its use although hints at using caution in its use especially in field soils and contaminated cultures. The background amounts of fatty acid 16:1ω5c in soil due to other microorganisms is a distinct possibility and a word of caution was therefore given by Olsson *et al.* in 1995 and in some of his subsequent studies.

In the process of development of fatty acid techniques, a significant step was to detect the association of fatty acids other than the 16:1ω5c with AM fungi. For example, fatty acids 16:0, 18:1ω7c, 18:1ω9c, 20:1ω9c, 20:3, 20:4, 20:5 have also been detected either exclusively or in higher amounts in AM structures (Graham *et al.* 1995; Olsson *et al.* 1995; Bentivenga & Morton 1996; Jansa *et al.* 1999; Madan *et al.* 2002). However all of these could not be considered as markers for AM fungi as they were also the major constituents of saprophytic fungi (Dembitsky *et al.* 1993; Frostegard *et al.* 1993; Stahl & Klug 1996; Jansa *et al.* 1999). Fatty acid 16:0 is also a major constituent of plant roots (Wellburn *et al.* 1994). The presence of large quantities of 18:1ω9c, 20:1ω9c, 20:2ω6c were reported in *Gigaspora* spp. as compared to the other AM species in some studies (Graham *et al.* 1995; Bentivenga & Morton 1996; Madan *et al.* 2002), suggesting that these fatty acids may have potential as Gigaspora-specific biomarkers. Only 18:1ω9c has been reported in large quantities (20-40% of the total lipids) in saprophytic fungi (Stahl & Klug 1996), while the other two 20-C fatty acids have not been reported from saprophytic fungi, although long-chained fatty acids (≥20 C) have been reported from *Mortierella elongata* in significant amounts (Jansa *et al.* 1999).

Combinations of fatty acids have also been suggested to have importance in detection of species in a mixed rhizosphere. For example, Olsson *et al.* (1995) suggested using the combination of 16:1ω5c and 20:5 for the detection of *Glomus* and *Acaulospora* species in soil while Madan *et al.* (2002) suggested a combination of 18:1ω9c and 16:1ω5c to detect *Gigaspora* spp.

Fatty acid analysis has also been used to differentiate saprophytic fungi from AM fungi (Larsen *et al.* 1998) and to differentiate species of *Pythium* (Pankhurst *et al.* 2001). Fatty acid techniques have been successful in studying the interaction between AM fungi and saprophytic fungi (Larsen *et al.* 1998; Larsen & Bodker 2001), and to quantify the parasitic fungus *Aphanomyces euteiches* in pea roots (Larsen *et al.* 2000). These studies indicate the potential use of fatty acids for quantification of competitive interactions between different pathogens in the same host and for estimating energy costs of mycorrhizas in the host plant.

The main problem associated with fatty acid analysis is that FAME or PLFA profiles of whole soil communities are very complex (up to 50 fatty acids may be detected) and sophisticated statistical methods are required to interpret them (Cavigelli *et al.* 1995). However, there is considerable potential for the use of selected fatty acids as biomarkers for specific microbial groups, providing it can be confirmed that these fatty acids occur predominantly in the targeted microbial group and not in other groups or plant tissues.

Alterations in total and relative amounts of fatty acids during different steps in the separation and extraction procedures may also cause problems. Some fatty acids were found to be sensitive to the sieving and physical disturbance of the soil, eg. sieving resulted in a slight decrease in the fatty acid 18:2ω6c, while a higher temperature (25°C) resulted in the decrease in total amount of PLFAs detected (Peterson & Klug 1994). The amount of PLFAs detected was also reduced by 21-54% during a 24-h chloroform fumigation (Peterson *et al.* 1991) period.

Application of fatty acids analysis to the detection and quantification of AM fungi is comparatively recent and our knowledge of the specific fatty acids produced by different species of AM fungi is still inadequate. We do not know the extent to which different AM species differ on the basis of their fatty acids profiles and if such profiles could be used to detect or differentiate specific species of AM fungi. Also,

although PLFA analysis has been used to estimate the biomass of AM fungal propagules in soil and plant roots (Olsson *et al.* 1995; Olsson *et al.* 1999; Larsen *et al.* 2000), this approach needs to be extended to a wider range of AM species. Application of the FAME and / or PLFA technique to detection and quantification of AM infectivity in field soils also needs to be explored.

1.4 Conclusions

In concluding this literature review, it can be stated that AM fungi are important for the growth plants in most natural and man-made ecosystems, with maintenance of AM infectivity of the soil being important for the sustainability of these ecosystems. Conventional methods of measuring AM infectivity are either limited or labour oriented, hence, alternative biochemical or molecular methods are required. Although some of these biochemical methods have shown promise, they are in their early stages of development and more research is needed to confirm their utility.

1.5 Project Aim

The overall aim of this project was to assess the role of specific fatty acids in studying AM fungi and to determine whether or not these specific fatty acids could be used to detect, identify and quantify the biomass of AM fungal propagules in controlled environment experiments (pot experiments) and in the field.

To ensure the efficiency of the technique, some experiments were done to test the technique. For example, ten common uncolonised host plant roots were tested for AM-specific fatty acid, to rule out potential background profiles from the host roots or from rhizosphere microorganisms other than AM fungi. Profiles were also obtained of the varying number of spore samples and dilution levels of soil samples from the field in order to confirm the validity of the technique.

Two methods of fatty acid analysis were evaluated during the early part of the work in order to assess their utility and practical value. These were fatty acid analysis based on extraction of total lipids from soil and plant roots (FAME analysis) and fatty acid analysis based on extraction of phospholipids (PLFA) and neutral lipids (NLFA).

Specific aims of the project were (1) to compare the capacity of the FAME and PLFA / NLFA techniques to detect different AM species in soil and plant roots; (2) to compare estimates of AM fungal biomass in soil and plant roots based on fatty

acid analysis with estimates based on conventional methodology; (3) to determine the fatty acid composition of different components of AM fungi during the development of the plant-mycorrhizal association; (4) to compare the fatty acid composition and content of spores of different AM species; (5) to determine if the fatty acid analysis could be used to differentiate between AM fungal genera and between species within genera; and (6) to test the capacity of fatty acid analysis to provide a measure of AM infectivity in field soils.

Chapter 2: Material and Methods

This chapter describes the general materials and methods used frequently during the project. The specific methods used for some experiments are described in subsequent chapters.

2.1 Host and Fungal Isolates

Selected AM species were grown on the host plant *Allium porrum* L. cv. Musselburgh (leek) in all experiments. The mycorrhizal species selected for one or more experiments included *Glomus coronatum* Giovanetti (WUM 16, University of Western Australia collection), *Glomus microcarpum* Tulsane & Tulsane (DPI EM 8), *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (strain 5, DPI 5), *Gigaspora margarita* Becker & Hall (Dijon pre-BEG collection), *Gigaspora rosea* Nicolson & Schenk (BEG 9), *Gigaspora decipiens* Hall & Abbott (WUM 6, University of Western Australia collection) and *Scutellospora calospora* Walker & Sanders (WUM 12, University of Western Australia collection).

2.2 Soils

Two soils were used in the experiments. A mixture of Mallala soil and autoclaved sand (1:9 w/w) was used in experiments 1 and 2 (Chapters 3 and 4 respectively). This soil was collected near Mallala (40 km west of Adelaide) South Australia and had a pH (water) of 7.8 and a P content of 6.28 ppm (Colwell *et al.* 1963). Both the sand and soil were autoclaved at 121°C for 50 minutes and thoroughly dried prior to use. For the other experiments, a 10% Kuitpo soil/sand mix (1:9 w/w) was used, which was autoclaved in the same way as the Mallala soil. The Kuitpo soil was collected from the Kuitpo forest (50 km south east of Adelaide) South Australia. It had a pH (water) of 5.1 and a P content of 2.13 ppm.

2.3 Preparation of Pots and Inoculation

Pots of 1.4kg capacity were used for all experiments. Leek seeds were surface sterilised in a 3% sodium hypochlorite solution for 5 minutes. The seeds were rinsed 5 times with reverse osmosis (RO) purified water before germinating them on moist

filter papers in an incubator at 25°C for 48 hours. The pre-germinated seeds were then planted into soil/sand mix at the rate of 2 seeds per pot (unless described otherwise). Each pot was inoculated with 10 to 20 AM spores per seed (unless described otherwise for individual experiments). Non-mycorrhizal control pots were not inoculated. Different numbers of replicates were used in different experiments as mentioned in the individual chapters.

2.4 Growth Conditions and Maintenance of Pots

The leek plants (5 replicate pots per treatment unless stated otherwise), were grown in the 1.4-kg pots under glass house conditions where the daylight intensity was 350-500 $\mu mol\ m^{-2}s^{-1}$ and the temperature varied between 14-22°C. The pots were watered with deionised water 3 times a week and fertilised once a week with 10 ml of a modified Long-Ashton nutrient solution without P (Smith and Smith 1981). The nutrient solution consisted of 2 mM K_2SO_4, 1.5 mM $MgSO_4$, 4 mM $CaCl_2$, 8 mM $NaNO_3$ and micronutrients (all in mg l^{-1}) 2.86 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.22 $ZnSO_4.7H_2O$, 0.08 $CuSO_4.5H_2O$, 0.025 $Na_2MoO_4.2H_2O$ and Fe-EDTA to give 5 mg Fe l^{-1}.

2.5 Harvesting of Pots

Pots were harvested at 14 weeks, unless described otherwise with specific information in individual chapters. Soil was collected and placed in plastic bags and stored in a freezer until required. Plants were washed and the fresh weight of roots and shoots was measured. A sample of roots (0.1 g) was cut into 1cm pieces and placed in vials containing 10% KOH, as a clearing agent prior to staining. Roots not required for immediate analysis were stored in a freezer at –20°C.

2.6 Staining of Roots

A modified method of Phillips & Hayman (1970) was used to stain root pieces. Root pieces cleared in 10% KOH for 5 days were rinsed briefly in 0.1 HCl to neutralise the KOH. The root pieces were then stained with a lactoglycerol-based trypan blue stain (Appendix 1) for 20-40 min at room temperature. Excess stain was removed by washing twice with 0.05% lactoglycerol and the roots were then observed under a microscope.

2.7 Quantification of % Colonisation of Roots by AM Fungi

The line intercept method of Tennant (1975) was used to determine the % colonisation of roots by AM fungi. The root pieces were randomly spread on a grid so that they overlapped each other as little as possible. Counts were then made of the number of times a root intersected the grid and the number of intersects in which the root contained a mycorrhizal structure.

2.8 Spore Isolation and Counting

A 50 or 100 g soil sample was soaked in water in a beaker for 2h prior to wet sieving. The scum at the top of the beaker that contains dead spores was skimmed off, and rest of the suspension was passed through a 53-µm pore-sized mesh. The soil sample was washed several times and the washing passed through the sieve until the water was colorless. The residue in the sieve was then collected in a centrifuge tube with the help of water and centrifuged at 1800 rpm for 5 min. The upper phase containing dead spores was discarded. A small amount of 75% sugar solution was added to the lower phase (soil + spores) and the contents were centrifuged at 1800 rpm for 15 sec. The upper phase of sugar solution with spores was carefully passed through the sieve. The spores were then washed three times with RO water to remove excess sugar and counted in a nematode dish under a dissecting microscope at x50 magnification. A representative sample of spores, picked individually, presumably of different ages, was included in each sample.

2.9 FAME Analysis

Cellular and non-cellular fatty acids were extracted from soil, root and spore samples using the method developed by Microbial I.D. Inc. (MIDI), Newark, DE, for extraction of FAMEs (fatty acid methyl esters) from bacteria and used by Graham *et al.* (1995) for AM fungi. Root samples were frozen in liquid nitrogen and ground to a powder in a mortar and pestle before use. Spore sample sizes varied in different experiments as mentioned in individual Chapters. The soil (6 g), root (0.3 g) and spore samples (variable, described in each Chapter) were placed in glass test tubes with 6, 4 and 2 ml respectively of 3.75 M NaOH in 50% aqueous methanol (Reagent 1), and sealed with a Teflon-lined screw cap. The tubes were vortexed for 30 s and

then placed in a boiling water bath for 30 min. After cooling in ice water, the tubes were centrifuged at 3,000 *g* for 10 min. The supernatant (3, 2 and 1 g respectively) was transferred to a 15 ml glass tube and 6, 4 and 2 ml (respectively) of 3.25 M HCl in 45% aqueous methanol (Reagent 2), and 75 µl of an internal standard [nonadecaonic acid (19:0)] were added. The solutions were mixed on a vortex mixer for 10 s, heated at 80°C for 10 min, cooled and extracted with 1.5 ml of 1:1 hexane:methyl-tert-butyl ether (Reagent 3). The tubes were then centrifuged at 3,000 *g* for 4 min and the supernatant transferred to a fresh tube and washed once with 3 ml of 0.024 M NaOH (Reagent 4). The supernatant was then transferred to glass vial and evaporated under a stream of N_2 at room temperature. The residue was taken up with a known volume (100 or 300 µl) of Reagent 3 prior to gas chromatographic (GC) analysis (see below).

Details of the preparation of reagents 1-4 are given in Appendix 1.

2.10 PLFA / NLFA Analysis

Soil and root samples (ground in liquid N_2 to a fine powder) were subjected to lipid extraction and then fractionated into neutral lipids, glycolipids and phospholipids by the method used by Bossio and Scow (1998). Each sample was extracted overnight in 73.2 ml of one-phase buffer containing chloroform: methanol: phosphate buffer (1:2:0.8 by vol; pH 4.0). The extracts were then filtered through fast ash-less filter paper and split into two phases by adding 25 ml of chloroform and 25 ml of phosphate buffer to the soil extracts and 10 ml of chloroform and 10 ml of phosphate buffer to the root extracts. The $CHCl_3$ layer was decanted into a round-bottomed boiling flask and evaporated to dryness on a rotary evaporator at 32°C. The extracts were then resuspended in 3 ml of $CHCl_3$ and applied to a column of silicic acid conditioned with $CHCl_3$. Neutral lipids were then extracted with 10 ml chloroform, glycolipids with 20 ml acetone and phospholipids with 10 ml methanol. The neutral lipid and phospholipid fractions were dried under N_2 at 30°C. The internal standard (75 µl nonadecaonic acid) was then added to the samples, which were subjected to mild alkaline methanolysis (Bossio and Scow, 1998). Resulting FAMEs were dissolved in 150 µl hexane prior to GC analysis.

2.11 Gas Chromatography of Fatty Acids

FAMEs were separated by capillary gas chromatography using the automated procedure developed by MIDI. The system consists of a GC (HP 5890) - Hewlett Packard HP with flame ionisation detector, HP-IB communications, HP 3365 ChemStation software and computer. The HP 3365 ChemStation software operates the sampling, analysis and integration of the chromatographic sample. The GC was equipped with a HP 25 m x 0.2 mm fused silica capillary column. The temperature program was ramped from 170°C to 250°C at 5°C per minute. Hydrogen was the carrier gas. The FAME peaks were identified by the MIDI program based on their equivalent chain length. The peak areas were normalized against the 19:0 internal standard and expressed as percentage by weight. The nomenclature of fatty acids followed here is as described by Weete (1980).

2.12 Analysis of FAME data

Comparison of the abundance of individual FAMEs was made using two-way ANOVA. The differences in the abundance of the individual FAMEs were tested using Fisher's least-significant-difference procedure. FAME profiles were further analysed by canonical variate analysis (CVA). Data were analysed using GENSTAT 5 (Genstat 5 Committee, 1987).

Chapter 3: Fatty Acid Analysis of Five AM Fungal Species and Comparison of FAME, PLFA and NLFA Profiles

3.1 Introduction

The capacity to identify AM fungal species and measure the biomass of their propagules is an important pre-requisite for manipulation of mycorrhizal fungal populations in the soil for sustainable agriculture. As discussed in Chapter 1, microscopic techniques presently used neither differentiate AM fungal hyphae from the hyphae of other fungi, nor reliably separate viable fungal propagules from dead or non-viable propagules. Fatty acid analysis may be an alternative to the traditional techniques, although we lack specific knowledge of the fatty acids produced by various species of AM fungi and the extent to which the fatty acid profiles of AM fungi could be used to detect or differentiate different species. Graham *et al.* (1995) previously studied 53 taxa belonging to five families of Glomalean fungi, while Bentivenga & Morton (1996) explored inter specific differences for 17 taxa in the family Gigasporaceae. Both these studies found a lack of variation in the fatty acid profiles between the species within taxa. However, both studies were performed using fatty acids from total lipids. The different techniques of extracting fatty acids from either total lipids, phospholipids or neutral lipids may have a significant effect on the resulting fatty acid profiles as was reported by Olsson *et al.* (1995) in phospholipid and neutral lipid profiles of *Glomus* and *Acaulospora* spp. There are no reports of studies so far that compared the differences between the concurrent profiles of the same AM species using different techniques eg. FAME and PLFA / NLFA analyses.

The experiments discussed in this chapter were carried out with five AM fungal species of three AM genera, with the aim of finding specific fatty acids linked with these AM species. Fatty acid analysis was performed on total lipids, phospholipids and neutral lipids obtained from the spores of AM fungi, roots of the host plants and soils colonised by AM fungi, with a special aim of comparing the differences in profiles obtained from different AM component with two different

techniques. The objectives of this work were to evaluate the capacity of two methods of fatty acid analysis to (1) differentiate between the soil and the roots of leek plants colonised by five AM fungi and plants grown without mycorrhizal colonisation, (2) differentiate between spores, colonised roots and colonised soil of the five AM species, and (3) correlate the degree of AM fungal colonisation of plant roots with the amounts of selected fatty acids present. The two methods were (1) fatty acid analysis based on extraction of total ester linked fatty acid methyl esters (FAME) and (2) fatty acid analysis based on extraction of phospholipids-linked fatty acids (PLFA) and neutral-lipid-linked fatty acids (NLFA).

Furthermore, little is known of the fatty acids present in the roots of common AM host plants. The possibility of finding the key AM biomarker fatty acid 16:1ω5c in the roots of host plants is crucial in these experiments. This knowledge was even more significant since there were reports of the presence of fatty acid 16:1ω5c in some bacteria and saprophytic fungi (Nichols *et al.* 1986; Jansa *et al.* 1999). Moreover, variation in rhizosphere communities between species of host plants can also be another probability, which needed to be explored. In order to rule out the possibility of obtaining background profiles of the roots of AM host species plus their associated microorganisms, another small experiment was simultaneously carried out choosing the non-mycorrhizal roots of some commonly used host plants. The FAME profiles of the roots of ten different plant species (described in section 3.3.2), normally colonised by AM fungi, were examined for the presence of fatty acids of special interest.

The main experiment of obtaining fatty acid profiles of five AM species also looked at the possibility of the effect of P nutrition on the fatty acid profiles. The high P levels in soil are known to affect the growth of mycorrhiza adversely (Peng *et al.* 1993; Smith & Read 1997). Hence, two sets of non-mycorrhizal controls were used in the main experiment to evaluate the difference on fatty acid profiles in the presence and absence of P in soil. One set of controls was provided with P nutrition while the other set of controls, along with the mycorrhizal plants, received nutrition without P (Smith and Smith 1981).

3.2 Aims

The aims of this experiment were:

1. To obtain the fatty acid profiles of the spores of five AM species.
2. To differentiate uncolonised soil and roots of leek plants from soil and roots colonised with AM fungi using FAME, PLFA and NLFA profiles, and to determine the potential influence of P nutrition on the profiles.
3. To determine if the fatty acids associated with different AM species could be used to detect AM fungi or differentiate AM species.
4. To compare a microscopic method for measuring the biomass of AM fungi with a method based on the amount of a specific fatty acid detected.
5. To compare and evaluate the two techniques (FAME and PLFA / NLFA) of fatty acid analysis.
6. To confirm the absence of AM-specific fatty acids in non-colonised host roots by obtaining the fatty acid profiles of selected host plant species.

3.3 Materials and Methods

3.3.1 Experimental Design:

Five AM species (*Glomus coronatum*, *G. microcarpum*, *Gigaspora margarita*, *Gi. rosea* and *Scutellospora calospora*) were grown on the host plant (leek). The plants were grown in 1.4-kg pots in low-P soil under glass house conditions (Chapter 2). Two sets of non-mycorrhizal plants, one set with additional P as KH_2PO_4 at 1mM/kg soil (B+P) and the other set without additional P (B-P) were included. Each non-mycorrhizal and mycorrhizal treatment had five replicates. Planting was timed as follows:

All non-mycorrhizal plants were set up in the first week, followed by all five replicate pots of one species at weekly intervals. All plants were allowed to grow for 14 weeks and then harvested in the same order as inoculation. Hence, at the time of harvest all treatment plants were of the same age.

At each harvest the plants of each pot were removed and the fresh weight of their shoots and roots determined.

The soil from each pot was sampled as follows:

- 6 g soil collected in a glass tube for FAME analysis
- 12 g soil collected in a beaker for PLFA / NLFA analysis
- 100 g soil collected in a beaker for spore isolation

The roots of plants from each pot were sampled as follows:

- 0.15 g roots randomly selected for staining
- 0.3 g roots selected for FAME analysis
- 0.3 g roots selected for PLFA / NLFA analysis

The roots selected for FAME and PLFA / NLFA analysis were stored at –20°C for 2-8 weeks prior to processing.

3.3.2 Host Plants and Pot Cultures

FAME profiles were obtained from the roots of Canola (*Brassica napus* L.), Wheat (*Triticum aestivum* L.), Ryegrass (*Lolium multiflorum* Lam.), Marigold (*Calendula officinalis* L.), Plantago (*Plantago lanceolata* L.), Sorghum (*Sorghum moench* L.), Mung beans (*Vigna radiata* L.), Tomato (*Lycopersicum esculentum* Mill.), Onion (*Allium cepa* L.), Linseed (*Linum usitatissimum* L.) and Leek (*Allium porrum* L. cv. Musselburgh). These selected species were chosen to include a range of mycorrhizal dependency as well as a non-mycorrhizal species, Canola. These species were selected and studied because these are commonly used as AM host plants in pot experiments.

Sterilised sand was used as the growth medium to avoid contamination by microorganisms that would be present if soil was used. Small plastic containers were filled with 500 g of sand and were wrapped with aluminium foil to avoid the growth of algae. Seeds of the host plants were surface sterilised with a 3% sodium hypochlorite solution and thoroughly rinsed with sterile water before three seeds were placed in each pot. The pots were placed in the glass house, watered every day and fertilised once a week with 10 ml of a modified Long-Ashton nutrient solution (-P) (Smith & Smith 1981). Three pots of each host type were grown. Plants were harvested at the end of the growth period (14 weeks) and their roots were examined microscopically for signs of mycorrhizal fungi.

3.3.3 Isolation of AM Fungal Spores from Soil

Spores were isolated from a sample of 100 g soil of each treatment by the method as described in Chapter 2, section 2.8.

3.3.4 % Colonisation of Roots by AM fungi

A 0.1 g root sample from each replicate pot was used for estimating the percent of total root length colonised by AM fungi. The roots were stained as described in section 2.6 and % root length colonised measured as described in section 2.7 of Chapter 2.

3.3.5 FAME Analysis

FAME analysis was performed on soils, host roots and mycorrhizal spores using the protocol as described in section 2.9 of Chapter 2. The number of spores of each of the five AM species used for FAME analysis is shown in Table 3.1.

Table 3.1 Total weight (g) and number of spores obtained for FAME analysis.

Treatments	Total Fresh Weight of Spores (g)	Total Number of Spores (Estimated)
G. coronatum	0.10	455
G. microcarpum	0.38	9500
Gi. margarita	0.66	2357
Gi. rosea	0.58	2230
S. calospora	0.29	3625

3.3.6 PLFA and NLFA Analysis

A 12 g soil sample and a 0.3 g root sample from each replicate pot was used to obtain PLFA and NLFA profiles using the technique as described in section 2.10 of Chapter 2. No PLFA / NLFA analysis was performed on spores.

3.3.7 Statistical Analysis

The data obtained by FAME and PLFA / NLFA analysis was subjected to a number of statistical approaches. This included analysis of variance (ANOVA) of each fatty acid separately, which tested the level of significance of differences in the amount of individual fatty acids detected. However, ANOVA is not able to determine which combinations of fatty acids are most likely to be important in differentiating between different fatty acid profiles. Therefore a multivariate technique, canonical variate analysis (CVA), appropriate for replicated treatments, was included. This test has the ability to compare differences in population means for all variables (fatty acids) simultaneously and it finds the best combinations of variables that maximise the differences between groups. CVA chooses linear combinations of the fatty acids. The combination with the greatest variability between treatments is called the first canonical variate (CV1) and the second greatest variability among treatments as CV2 and so on. There is a necessary constraint that each canonical variate is uncorrelated with the preceding canonical variate. Ideally, it is hoped that the first two canonical variates would give an adequate representation of the best separation between groups. Some variables were log transformed to satisfy the assumptions of ANOVA and CVA. Data were analysed using GENSTAT 5 (Genstat 5 Committee, 1987).

3.4 Results

3.4.1 Plant Growth and Mycorrhizal Colonisation

The roots of all inoculated plants became colonised with AM fungi during the 14-week growth period, with % colonisation ranging from 15% for *S. calospora* to 62 % for *G. microcarpum* (Table 3.2). Non-inoculated plants did not become colonised. All plants inoculated with mycorrhizal fungi were larger than non-mycorrhizal plants without P, but only plants inoculated with *G. microcarpum* were larger than the non-mycorrhizal plants with added P (Table 3.2). The growth response to mycorrhizal inoculation was positively correlated with % root length colonised ($r^2 = 0.87$). Production of spores was greatest in the soil of the plants inoculated with *G. microcarpum* and lowest in the soil of plants inoculated with S. *calospora* (Table 3.2).

3.4.2 FAME Analysis of Spores

Six dominant fatty acids were detected in the spores of all five AM fungal species (Table 3.3). Fatty acid 16:1ω5c was the most abundant followed by 16:0, 18:1ω7c, 18:1ω9c, i17:1 and 18:2ω6c. The relative amount of 16:1ω5c ranged from 10.1% in the spores of *Gi. rosea* to 58.1% in the spores of *G. coronatum*. Eleven other fatty acids were detected in small amounts (Table 3.3). Of these, i20:0 was present only in the spores of *G. microcarpum*, 20:4ω6c only in the spores of *Gi. rosea* and 20:1 ω9c only in the spores of *Gi margarita* and *Gi. rosea*. Fatty acids 20:0, 22:0 and 22:1ω9c were present only in the spores of species of *Gigaspora* and *S. calospora* (Table 3.3).

Table 3.2. Average fresh plant weight (roots + shoots), % colonisation of roots and number of spores in the soil of experimental pots inoculated with five AM fungi (± SE, n = 5) after 14 weeks growth.

Plant / AM fungus combination	**% Colonisation of roots**	**Fresh Plant weight (roots + shoots) (g)**	**Number of spores / g soil**
B-P*	0	0.31 ± 0.01	0
B+P*	0	2.43 ± 0.06	0
Glomus coronatum	44.8 ±2.4	1.86 ± 0.09	0.6 ± 0.03
G. microcarpum	62 ± 1.4	3.39 ± 0.16	1.68 ± 0.16
Gigaspora margarita	33.5 ±5.1	0.96 ± 0.16	0.26 ± 0.02
Gi. rosea	30.6 ± 3.4	0.51 ±0.04	0.71 ± 0.06
Scutellospora calospora	14.9 ± 0.23	0.39 ± 0.03	0.24 ± 0.01

*B-P = non-mycorrhizal control without P, B+P = non-mycorrhizal control with P.

Table 3.3. Relative amounts (weight %) of selected fatty acids detected in FAME profiles of the spores of five AM fungal species. Values obtained from a single sample of varying amounts of spores (Table 3.1) and represent only a selected groups of fatty acids. The remaining % was made up of fatty acids, which individually contributed less than 1% of total fatty acids (not described).

Fatty Acids	*G. coronatum*	*G. microcarpum*	*Gi. margarita*	*Gi. rosea*	*S. calospora*
			(weight %)		
16:0	21.3	28.5	21.9	21.3	20.9
16:0 2OH	0	0	0.4	0.4	1.0
16:1ω5c	58.1	50.9	42.8	10.1	10.4
i17:1	1.1	1.6	1.5	1.3	5.1
18:1ω7c	8.2	5.0	7.2	5.9	3.3
18:1ω9c	2.4	3.6	4.6	24.3	5.9
18:2ω6c	1.6	1.2	1.9	1.9	1.6
18:3ω6c	0.2	0.2	0.3	0.4	0.9
20:0	0	0	0.3	0.4	1.2
i20:0	0	2.2	0	0	0
20:1ω9c	0	0	0.5	11.1	0
20:2ω6c	0.2	0	0.3	2.2	0
20:3ω6c	0.9	0.2	0.5	1.5	0.7
20:4ω6c	0	0	0	0.5	0
i21:01	0.2	0.6	1.7	0.8	3.6
22:1ω9c	0	0	0.4	1.6	1.1
22:0	0	0	0.3	0.2	1.8

3.4.3 FAME, PLFA and NLFA Analysis of Roots

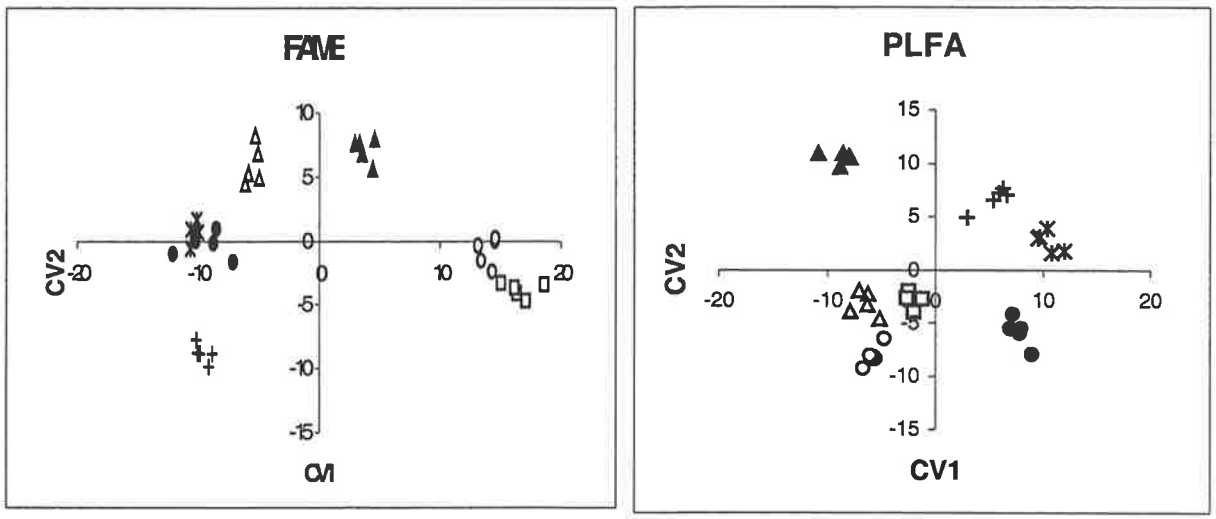
A summary of the major and discriminating fatty acids obtained from the FAME, PLFA and NLFA profiles of the roots of the mycorrhizal and non-mycorrhizal plants is given in Table 3.4. The major fatty acids present in all three sets of profiles were 16:0 and 18:2ω6c. Other major fatty acids were 21:0 and C21 primary alcohol (FAME), 18:1ω9c (PLFA), and three fatty acids (C16 N Alcohol, unknown C22.258 and unknown C23.676) (NLFA). Several fatty acids detected in the mycorrhizal roots were either not detected or were present in very low amounts in the non-mycorrhizal roots. These included 16:1ω5c, 18:1ω9c, 21:0 2OH and 22:0 (FAME), 16:1ω5c, i17:1 and 20:0 (PLFA), and C16 N Alcohol, 15:0 2OH, 16:1ω5c and 22:1ω9c (NLFA) (Table 3.4). Fatty acid 16:1ω5c was the only fatty acid showing discrimination between mycorrhizal and non-mycorrhizal roots in all three analyses and even in this case not all mycorrhizal fungi appeared to produce this fatty acid in roots. There were also some marked differences between individual AM fungal species with some of the fatty acids detected. These included large relative amounts of 16:1ω5c in roots colonised by *G. microcarpum* (FAME, PLFA), large relative amounts of 15:0 2OH in roots colonised by *Gi. margarita* (PLFA and NLFA), and large relative amounts of 22:1ω9c in roots colonised by *G. coronatum* (NLFA).

Canonical variate analysis (CVA) of the FAME, PLFA and NLFA profiles of the roots is given in Figure 3.1. The amount of variation explained by the first two co-variates in these analyses was 81.1% (FAME), 86.8% (PLFA) and 89.6% (NLFA). Discrimination between the mycorrhizal and non-mycorrhizal roots was greatest with the FAME CVA (Figure 3.1a), whereas separation of the five different AM mycorrhizal root treatments was marginally better with both the PLFA and NLFA CVA (Figs. 3.1b, 1c). All three CVAs showed discrimination between the roots colonised by *G. coronatum, G. microcarpum, Gi. margarita* and *Gi. rosea* but the FAME CVA did not distinguish between the *S. calospora* and the *Gi. margarita* colonised roots (Figure 3.1a).

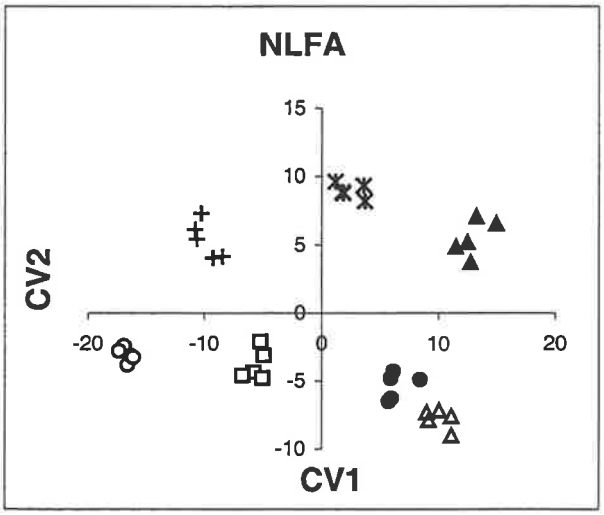
Table 3.4. Relative amounts (weight %) of selected fatty acids detected in FAME, PLFA and NLFA profiles of the roots of leek plants colonised by five different AM fungi. (The values are the means of five replicates of each treatment, B-P = non-mycorrhizal plant without P, B+P = non-mycorrhizal plant with P, GC = *G. coronatum*, GMIC = *G. microcarpum*, GM = *Gi. margarita*, GR = *Gi. rosea*, SC = *S. calospora*).

Fatty acid (weight %)	**B-P**	**B+P**	**GC**	**GMIC**	**GM**	**GR**	**SC**	**LSD *P*<0.05**	**Signifi cance**
FAME									
16:0	14.6	17.4	13.2	17.7	13.3	17.0	12.8	3.9	*
16:0 2OH	0.14	0	6.12	0.36	0.18	0	0.10	0.2	*
16:1ω5c	0	0	1.21	5.12	1.92	0.15	0	1.5	***
18:1ω9c	0	0	1.9	1.5	0	0	0.65	0.7	***
18:1ω9t	4.8	5.9	5.9	5.9	5.1	11.2	4.1	3.0	***
18:2ω6c	23.4	27.1	29.5	35.0	29.4	19.9	19.8	7.7	**
20:0	0.98	0.94	0.33	0.46	0.53	0.28	0.33	0.5	*
21:0	16.8	20.5	14.7	10.4	14.8	15.3	20.8	3.5	***
21:0 2OH	0	0	0.5	0.13	0.41	0.17	0.63	0.2	***
C21 Pr Alcohol	20.5	18.2	17.2	11.7	18.0	18.2	25.5	6.5	**
22:0	0	0	0.51	0.54	0.91	0.67	0.71	0.5	**
PLFA									
16:0	22.3	28.2	18.5	26.7	21.8	15.6	35.6	2.19	***
15:0 2OH	0	0.98	0	1.77	11.6	0	0	1.04	***
16:1ω5c	0	0.03	0.46	0.82	0.58	0	0	0.25	**
i17:1	0	0	3.63	1.18	3.09	1.60	2.89	0.71	***
18:1ω9c	6.76	4.8	6.23	9.28	4.43	4.15	5.12	1.05	***
18:2ω6c	37.5	52.6	39.3	43.2	26.6	38.5	29.2	4.02	***
20:0	0	0	0.31	1.09	1.49	0.13	0	0.34	***
22:0	2.98	0.61	0.82	0.97	0.92	1.06	0	1.59	ns
NLFA									
C16 N Alcohol	0	0	6.77	4.97	7.79	2.83	5.54	0.99	***
16:0	1.25	3.01	3.20	4.11	3.85	3.25	3.59	0.95	ns
15:0 2OH	0	0	0	0.16	1.22	0	0	0.20	***
16:1ω5c	0	0	0.49	0	0.96	0.27	0	0.37	ns
i17:1	0.62	13.4	3.47	1.42	6.88	0.24	3.03	2.37	***
18:2ω6c	6.84	6.46	5.88	7.78	3.75	1.99	4.49	1.21	***
20:0	0	0.85	0	0.26	0	0	0	0.14	***
21:0	3.88	1.73	0	0	0	2.67	0	0.40	***
22:1ω9c	0	0	16.7	2.03	6.89	8.13	0	1.19	***
C22.258	26.9	14.0	19.9	10.3	2.50	28.3	27.8	6.88	**
C23.676	0	15.8	19.6	16.0	10.4	8.42	19.5	2.23	***

***P <0.001, ** P <0.01, * P <0.05, ns = not significant



(a) (b)



(c)

Figure 3.1. Canonical variate analysis of FAME (a), PLFA (b) and NLFA (c) profiles of the roots of mycorrhizal and non-mycorrhizal treatments. O (B-P), □(B+P), ▲ (*G. coronatum*), Δ (*G. microcarpum*), ✳ (*Gi. margarita*), + (*Gi. rosea*) and ● (*S. calospora*).

3.4.4 FAME, PLFA and NLFA Analysis of Soils

A summary of the major and most discriminating fatty acids obtained from the FAME, PLFA and NLFA profiles of the soil removed from under the mycorrhizal and non-mycorrhizal plants is given in Table 3.5. Major fatty acids detected were 14:0, 16:0, 16:1ω5c, i17:1, 18:1ω9t and 18:2ω6c (FAME), 14:1cis7, a15:0, 16:0, 18:1ω9c, 18:1ω9t, 18:2ω6c and unknown C16.294 (PLFA), and C16 N Alcohol and 18:3ω6c (NLFA). Major discriminating fatty acids that were present in soil under mycorrhizal plants but absent in soil under non-mycorrhizal plants were i17:1 and unknown C22.271 (FAME), 15:0 2OH (PLFA) and 15:0 2OH, 16:1ω7c, 20:0 3OH, 21:1ω7c, unknown C21.688 and unknown C23.269 (NLFA). There were also significant differences in the amount of several fatty acids in soil under plants colonised by the different AM fungal species. These included 16:1ω5c, i17:1 and 18:1ω9t (FAME), 14:1cis7, 15:0 2OH, 16:1ω5c, unknown C16.294 and 18:1ω9t (PLFA), 15:0 2OH, 16:1ω5c, 16:1ω7c and all 20-C fatty acids (NLFA) (Table 3.5).

Canonical variate analysis (CVA) of the FAME, PLFA and NLFA profiles of the soil are given in Figure 3.2. The amount of variation explained by the first two co-variates in these analyses was 86.8% (FAME), 83% (PLFA) and 84.5% (NLFA). The FAME and the PLFA CVA showed discrimination between the soils under the mycorrhizal plants and the non-mycorrhizal plants. However, only the PLFA CVA (Figure 3.2b) separated the soils for each of the mycorrhizal treatments, with strong differentiation between the soils of the *G. coronatum* and *G. microcarpum* colonised plants and between the soils of the *Gi. margarita* and *Gi rosea* colonised plants. In contrast, the FAME CVA (Figure3.2a) showed no discrimination between the soils from each of these treatments, whilst the NLFA CVA (Figure 3.2c) showed good separation between the soils of the *Gi. margarita* and *Gi. rosea* colonised plants only. The PLFA and NLFA profiles of the soil from under the *S. calospora* colonised plants grouped closely with those of the soil from under the *G. coronatum* and *Gi. rosea* colonised plants.

Table 3.5. Relative amounts (weight %) of selected fatty acids detected in FAME, PLFA and NLFA profiles of soil under leek plants colonised by five different AM fungi. (The values are the mean of five replicates of each treatment, B-P = non-mycorrhizal plants without P, B+P = non-mycorrhizal plant with P, GC = *G. coronatum*, GMIC = *G. microcarpum*, GM = *Gi. margarita*, GR = *Gi. rosea*, SC = *S. calospora*). [*** P <0.001, ** P <0.01, * P <0.05, ns = not significant]

Fatty Acid (weight %)	B-P	B+P	GC	GMIC	GM	GR	SC	LSD (P<0.05)	Signifi cance
FAME									
12:0	2.35	2.36	1.94	0.87	1.60	1.98	1.43	0.75	**
14:0	7.79	6.25	3.58	2.20	3.71	3.76	3.88	2.38	***
a15:0	1.67	2.97	1.50	1.08	1.73	1.64	2.03	0.69	***
16:0	13.1	19.6	14.8	11.7	20.0	17.7	17.8	5.52	*
16:1ω5c	3.54	3.75	16.4	10.1	12.7	3.15	2.90	7.36	**
16:1ω7c	2.81	3.97	3.0	1.45	3.32	3.23	2.75	1.08	**
16:1ω9c	1.76	2.42	0.10	0.64	1.91	2.52	2.19	1.21	**
i17:1	0	0	8.59	6.48	0	0	8.24	2.76	**
18:1ω9c	3.19	5.32	4.25	2.38	5.52	4.10	4.73	1.39	***
18:1ω9t	7.32	12.1	4.86	4.12	10.9	12.9	16.2	4.35	***
18:2ω6c	2.56	4.60	2.34	2.33	4.98	7.39	9.26	3.21	***
18:3ω6c	1.04	2.00	1.49	0.76	1.53	1.86	1.71	0.81	*
21:0	5.13	6.12	4.64	4.49	5.07	5.40	8.0	3.02	ns
C21 Pr Alcohol	5.01	4.92	4.33	5.08	5.07	5.05	4.34	2.42	ns
C18N Alcohol	0.19	0.23	0.91	2.49	0	1.04	0.90	1.12	**
C22.271	0	0	5.60	2.43	0	0	0	3.37	***
PLFA									
14:0	0.99	0.43	9.11	0.94	0	0	3.03	3.54	ns
14:1cis7	1.60	2.14	8.60	3.57	11.8	1.64	1.71	2.88	***
a15:0	7.94	1.98	1.54	1.77	0	0	2.81	2.52	ns
15:0 2OH	0	0	0.17	14.9	31.9	0	1.37	2.61	***
16:0	15.1	18.3	16.1	11.1	31.9	13.4	27.4	3.54	***
16:1ω5c	1.25	0.97	3.05	2.93	0	0	1.26	0.54	***
C16.294	6.35	13.1	13.3	0	0	7.50	7.80	2.33	***
i17:1	3.89	0	1.58	0.12	0	0	1.99	0.52	***
18:1ω9c	7.09	5.69	5.89	6.79	4.08	4.85	6.45	1.10	*
18:1ω9t	6.0	5.03	5.42	8.10	0.93	5.29	7.02	0.93	***
18:2ω6c	3.42	11.34	6.98	9.06	3.15	5.24	3.77	1.65	***
18:3ω6c	3.34	2.81	3.14	3.61	6.54	3.03	2.10	0.63	***
C18.307	2.40	4.09	3.27	3.09	7.75	2.05	3.65	1.0	***
NLFA									
15:0 2OH	0	0	0	2.21	1.63	0	0	0.52	***
C16 N Alcohol	0.95	4.77	9.91	1.70	1.41	3.03	10.11	1.15	***
16: 0	0.81	0.77	1.74	5.44	0	0.33	2.39	0.86	***
16:1ω5c	0	0.04	2.47	0	0	0.36	0	0.27	***
16:1ω7c	0	0	1.25	0	0	0.07	0.20	0.13	***
18:2ω6c	2.09	1.92	1.58	1.91	0	0.78	0.85	0.69	*
18:3ω6c	1.17	3.69	5.20	4.33	8.96	3.22	4.66	0.92	***
20:0 3OH	0	0	0	0	0.93	0.35	0	0.29	*
21:1 ω7c	0	0	0	0	3.83	1.04	0	0.39	***
23:0	1.0	0.01	0	0	3.09	0.65	0.1	0.48	***
C21 Pr Alcohol	0	0.04	0	3.67	0	0.45	0	0.50	***
C21.688	0	0	0	0	3.42	1.32	0	0.29	***
C23.269	0	0	0.75	0	9.69	2.39	0	0.79	***

3.4.5 Relationships between Colonisation of Roots by AM Fungi and Fatty Acid Composition of Roots and Soil.

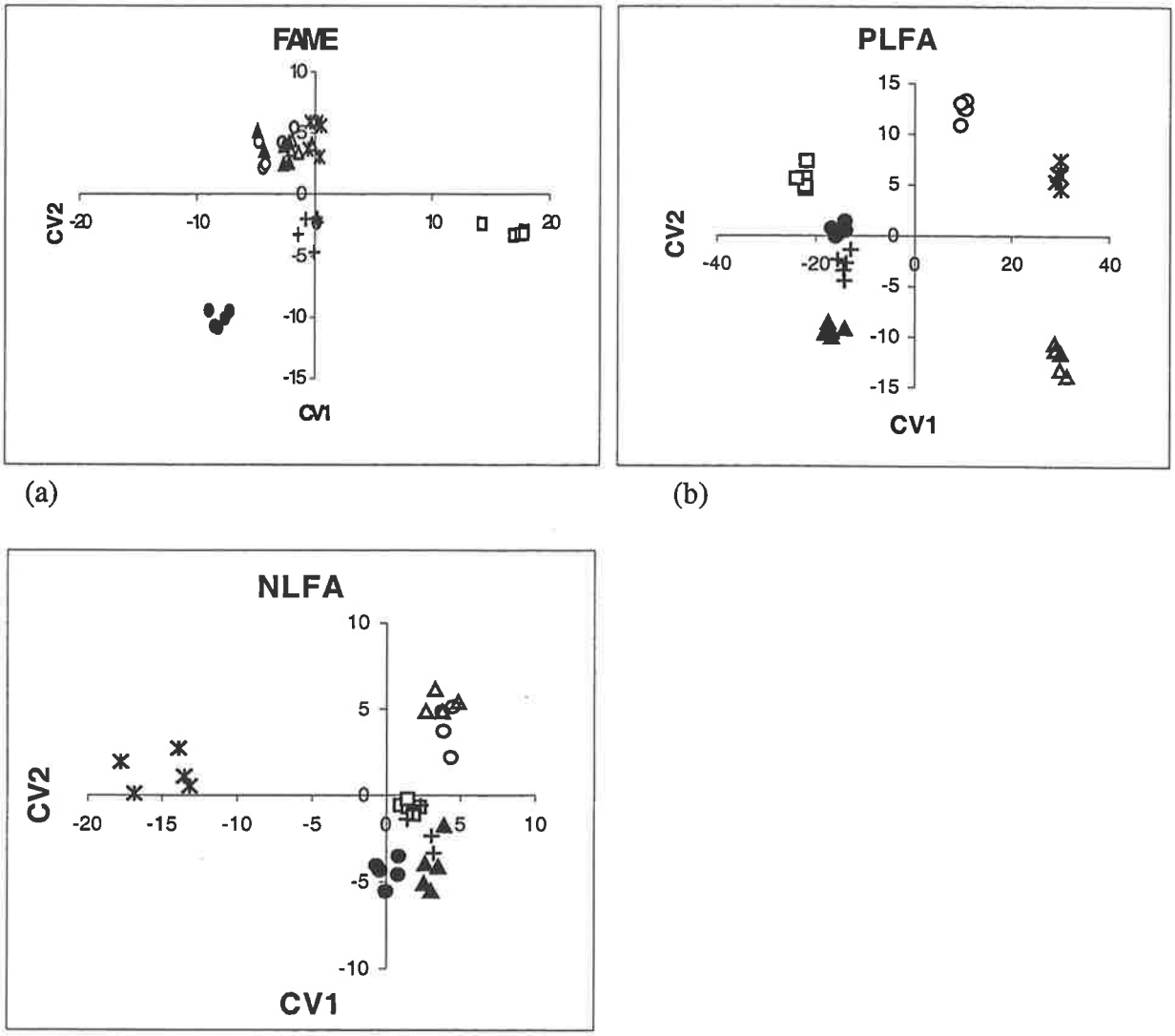
A regression analysis was carried out on the combined data for % colonisation of roots by the 5 different AM fungi and the relative amount of selected fatty acids present in roots and soil. Only fatty acid 16:1ω5c was found to have a positive correlation with these parameters (Figure 3.3 a, b, c). Positive correlations included r^2 values of 0.65 and 0.42 between % colonisation of roots and the relative amount of 16:1ω5c (FAME) in roots and soil respectively (Figure 3.3 a, c) and an r^2 value of 0.43 between % colonisation of roots and the relative amount of 16:1ω5c (PLFA) in roots. There was no relationship between % colonisation of roots and 16:1ω5c (PLFA) in soil or between % colonisation of roots and 16:1ω5c (NLFA) in roots or soil (results not shown). A positive but weak relationship ($r^2 = 0.34$) was also found between the number of spores per gram of soil and the relative amount of 16:1ω5c (FAME) in roots (Figure 3.3 d).

3.4.6 Microscopic Observations of Host Plant Roots

One sample of the roots of each non-inoculated host plant was examined under the microscope for the presence of mycorrhizal structures. No mycorrhizal colonization was observed in any of the samples (data not shown).

3.4.7 FAME Analysis of the Roots of Different Host Plant Species

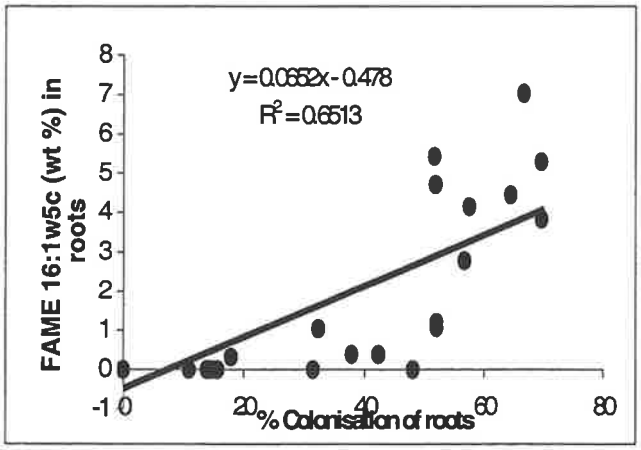
The relative amounts of selected fatty acids obtained from the roots of the different host plants using FAME analysis are shown in Table 3.6. None of the mycorrhiza-related fatty acids (16:1ω5c, 20:1ω9c) were present in the roots of the host plants. However, fatty acids 16:0, 16:1ω7c, 18:1ω9c and 18:2ω6c were present in most host plants in varying amounts (Table 3.6).



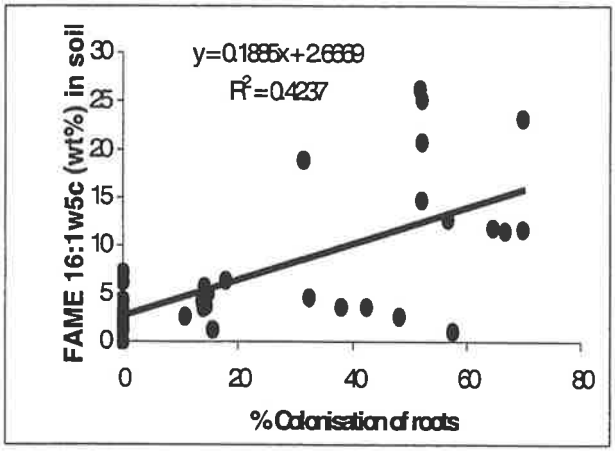
(a) (b)

(c)

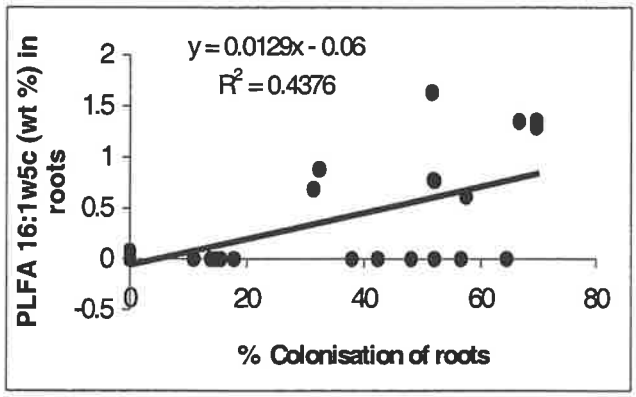
Figure 3.2. Canonical variate analysis of FAME (a), PLFA (b) and NLFA (c) profiles of the soil of mycorrhizal and non-mycorrhizal treatments. O (B-P), □(B+P), ▲ (*G. coronatum*), Δ (*G. microcarpum*), ✳ (*Gi. margarita*), + (*Gi. rosea*) and ● (*S. calospora*). Note change of scale.



(a)



(b)



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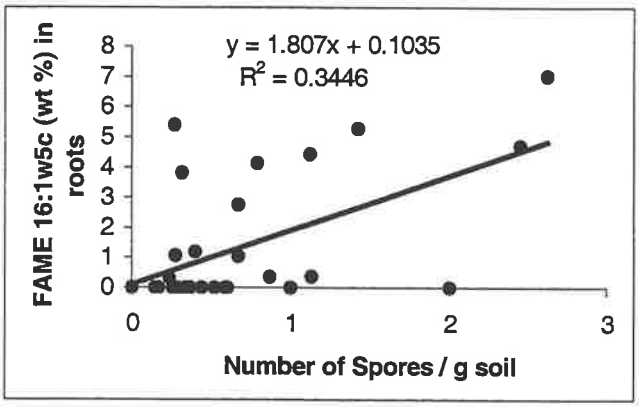


(d)

Figure 3.3 Regression analyses between % colonisation of roots by five different AM fungi and (a) FAME 16:1ω5c in roots, (b) FAME 16:1ω5c in soil, (c) PLFA 16:1ω5c in roots. The regression analysis between the number of spores of the five different AM fungi detected in the soil and FAME 16:1ω5c in roots in shown in (d).

Table 3.6 Relative amounts (weight %) of selected fatty acids obtained from 10 host plants by FAME analysis. Each value is a mean of three replicates. Values for individual fatty acids (ie.within rows) are significantly different at $p < 0.05$.

	Onion	Mungbean	Tomato	Ryegrass	Canola	Leek	Marigold	Sorghum	Linseed	Wheat
16:1ω9c	0	0	0	0	0.30	0	0	0	0	0
16:1ω7c	0	0.15	0	0.31	0.34	0	0	0.41	0	0
16:0	0	7.23	7.31	8.31	4.43	11.59	7.09	9.14	5.50	1.87
18:3ω6c	9.26	3.47	6.96	10.25	5.02	3.39	3.89	5.36	6.52	6.91
18:2ω6c	2.55	7.24	8.49	10.69	4.80	26.25	8.90	12.22	4.14	3.32
18:1ω9c	0	7.03	5.27	9.56	13.69	4.33	3.80	4.36	0	2.14
19:2ω6c	23.35	0.86	3.00	4.86	1.75	2.30	0.55	2.14	0	4.43
19:1ω8t	0	7.17	0.96	1.49	4.12	0.85	21.53	15.31	0	0
C20N Alcohol	16.86	12.33	23.05	17.52	9.76	4.89	8.13	10.04	0	2.30
C22 Pr Alcohol	15.27	14.53	22.29	9.60	9.37	7.64	9.65	12.62	13.48	8.80
C23 Pr Alcohol	0.00	0.43	1.23	0.21	1.18	1.06	0.00	0.00	0.00	1.94

3.5 Discussion

The major aim of this experiment was to obtain fatty acid profiles of various sources of five AM fungi (spores, roots and soils) using two fatty acid extraction techniques. Although previous studies have shown an association of some fatty acids, eg.16:1ω5c with AM fungi, particularly *Glomus* spp, there are few details of other fatty acids that may be linked to other AM species. The results of this experiment successfully correlated fatty acid 16:1ω5c with all AM fungi tested, and in addition identified other fatty acids (eg. 20:1ω9c, 22:1ω9c and 22:0) that could differentiate between individual AM species. The positive correlation of 16:1ω5c with plant growth and % colonisation of roots, confirmed a good agreement between fatty acid analysis and a morphological technique for estimating the amount of colonisation of plant roots by AM fungi. Furthermore, the experiment with different plant species confirmed that fatty acids 16:1ω5c and 20:1ω9c were absent from non-mycorrhizal roots and apparently are exclusively produced by roots colonised with mycorrhizal fungi. The details of the objectives and their achievement through this study are discussed below.

3.5.1 Capacity of FAME, PLFA and NLFA to Detect and Differentiate Five AM species

3.5.1.1. Spores

Sufficient spores were only available for the FAME analysis. The results (Table 3.3) showed that whilst the spores of the 5 different AM fungal species contained several common fatty acids a number of other fatty acids were detected that could be used to differentiate between individual species. The dominant fatty acid in the spores of 3 of the species (*G. coronatum, G. microcarpum* and *Gi. margarita*) was 16:1ω5c, which has been reported widely in AM fungi, particularly *Glomus* spp. (Jabaji-Hare 1988; Graham *et al.* 1995; Olsson *et al.* 1995; Jansa *et al.* 1999; Madan *et al.* 2002). Its presence in large relative amounts (42.8%) in *Gi. margarita* contrasts with the low amount reported previously for the spores of this species (Jabaji-Hare 1988). Whilst this fatty acid has potential to be used as a biomarker fatty acid for AM fungi (Olsson 1999) it may be less useful for differentiating between individual AM fungi. Similarly, the occurrence of other fatty acids present in the spores of all species in large but varying amounts (eg. 16:0, 18:1ω7c and 18:1ω9c) (Table 3.3) probably have limited value for diagnostic purposes. Fatty acid 18:1ω9c was higher in the spores of

the members of the Gigasporaceae (both species of *Gigaspora* and *S. calospora*) than in the spores of the two *Glomus* species, in agreement with the observations of Graham *et al.* (1995) and Larsen *et al.* (1998). In contrast to the 18-C length fatty acids, long-chain fatty acids (≥ 20-C) could be useful for differentiating between species. These include fatty acids 20:1ω9c, 22:1ω9c and 22:0 which differentiate the spores of *Glomus* spp. from those of *Gigaspora* spp., and i20:0 which differentiates *G. coronatum* from *G. microcarpum* (Table 3.3). Long-chain fatty acids are uncommon in saprophytic fungi (Stahl and Klug 1996) but have been reported previously in AM fungi (Jabaji-Hare 1988; Bentivenga and Morton 1994a; Graham *et al.* 1995), including the presence of 20:1ω9c in the spores of *Gi. margarita* (Madan *et al.* 2002).

3.5.1.2 Plant Roots

In plant roots, 16:1ω5c was the only fatty acid showing discrimination between mycorrhizal and non-mycorrhizal roots in the FAME, PLFA and NLFA profiles (Table 3.4). Furthermore it was the only fatty acid where the relative amount detected by the FAME or PLFA analysis was positively correlated with the % colonisation of the roots (Figure 3.3) although this correlation was weak. The reason for a weak correlation may be explained by the timing of the study, which was done after 14 weeks of growth by which time the amount of the fungus may have been decreased leading to the relatively weak correlation. This fatty acid is not normally found in plant roots (Olsson 1999) and is found in only low amounts (<5% of the total lipid present) in a few other fungi e.g. *Mucor* spp. (Jansa *et al.* 1999) and bacteria eg. *Flexibacter* (Nichols *et al.* 1986). It thus has potential as a biomarker to detect the presence of AM fungi in plant roots. However, in AM fungi such as *Gi. rosea* and *S. calospora* where the relative amount of 16:1ω5c in spores was found to be comparatively low (10%, Table 3.3), colonisation of plant roots by these fungi may need to be 30% or higher before this fatty acid is detectable. Other fatty acids that were detected in the mycorrhizal roots and either not detected or present in very low amounts in the non-mycorrhizal roots were either not present in all 3 fatty acid analyses (eg. 15:0 2OH and i17:1 were present in the PLFA and NLFA analyses but not in the FAME analysis), or where present in all 3 analyses (eg. 20:0) were inconsistently detected in mycorrhizal and non-mycorrhizal roots. Such fatty acids have limited general diagnostic potential but may be useful for discriminating

between particular species in experimental systems and where one form of fatty acid analysis is used. For example, with a PLFA analysis, the high relative amounts of 15:0 2OH in *Gi. margarita*-colonised roots may be useful to distinguish colonisation by this AM fungal species from colonisation by *Glomus* or *Scutellospora* spp. Generally speaking the long-chain (≥ 20-C) fatty acids potentially useful for differentiating the spores of AM species (Table 3.3) were inconsistently detected in the fatty acid profiles of the roots.

3.5.1.3 Soils

Fatty acid analysis of the soil associated with mycorrhizal and non-mycorrhizal plants also demonstrated the potential usefulness of 16:1ω5c as a biomarker for AM fungi. Its detection was again superior with the FAME analysis and as shown in Figure 3.3, there was a positive correlation between the amount of this fatty acid detected in the soil and the % colonisation of roots by the different AM fungi used in the experiment. Olsson *et al.* (1995) have also demonstrated a positive correlation between PLFA 16:1ω5c and the length of mycorrhizal hyphae in the soil, emphasising the potential usefulness of this fatty acid for the detection and estimation of the biomass of AM fungi in soil.

A high amount of FAME 16:0 from the spores, roots and soils of all inoculated treatments indicated that this fatty acid is also associated with all the AM fungal species included. However, it is also commonly reported from ectomycorrhizal and saprophytic fungi (eg. *Suillus variegatus*; Müller *et al.* 1994; also Jabaji-Hare 1988; Stahl and Klug 1996), other AM fungi (*Glomus intraradices*; Larsen *et al.* 1998), plant roots (Wellburn *et al.* 1994, and this study, see Table 3.6) and bacteria (Jarvis *et al.* 1996, Raffel *et al.* 1996; Thompson *et al.* 1993). Because of its universal presence in a variety of tissues, 16:0 has limited value as indicator of AM fungi.

It was observed that the types or amounts of fatty acids could vary depending on the source. For example, the amounts of fatty acid 16:1ω5c and 18:1ω9c were high in spores compared with root or soil samples associated with the same AM species (Table 3.7). FAMEs 20:1ω9c and C16 N Alcohol differentiated *Gigaspora* from the other two species at the spore level but were not detected in roots or soil samples. Similarly, FAME 22:1ω9c discriminated between Gigasporaceae and Glomaceae at the spore level but was not detected in root or soil samples (Table 3.7).

The absence of some specific fatty acids from roots and soils with containing AM structures suggests that these fatty acids may be present in storage structures. When storage structures were at low densities these fatty acids were not detectable. However, the diagnostic potential of these fatty acids cannot be underestimated either at taxon level or family level. The source-based differential information obtained from fatty acids suggested that the source from where the fatty acids are obtained is an important feature to consider when applying fatty acid analysis to AM fungi.

3.5.2 Comparison of the Fatty Acid Techniques

One objective of this study was to compare the capacity of two methods of fatty acid analysis to differentiate between the soil and the roots of mycorrhizal and non-mycorrhizal leek plants. As expected, the fatty acid profiles produced from extraction of total lipids (FAME) from plant roots or soil differed from those produced from the phospholipid (PLFA) and neutral lipid (NLFA) fractions. These differences were associated not only with the range of fatty acids detected in the profiles but also with the relative amounts of individual fatty acids detected (Tables 3.4 and 3.5). The capacity of the FAME, PLFA and NLFA profiles to differentiate between the soil and roots of the mycorrhizal and non-mycorrhizal leek plants, was tested by comparison of the canonical variate analysis of the profiles (Figures 3.1 and 3.2). This analysis showed that the FAME profiles gave better differentiation between the roots of mycorrhizal and non-mycorrhizal treatments whereas the PLFA and NLFA profiles were slightly superior to the FAME profiles in differentiating between roots colonised by the five different AM fungal species used (Figure 3.1). In contrast only the PLFA profiles gave good differentiation between the soils associated with the mycorrhizal and non-mycorrhizal treatments and between the soils associated with each AM fungal species (Figure 3.2).

The FAME and NLFA profiles are derived from fatty acids extracted from cellular and non-cellular lipids and in soil extracts. This will include non-cellular lipids derived from organic matter constituents (Zelles 1999). It was not unexpected therefore to see the discriminating power of the FAME and NLFA profiles of the soil to be less than that of the respective profiles derived from the plant roots. In contrast, the PLFA profiles are made up of fatty acids derived from the phospholipids of cellular membranes of plant cells and microorganisms (Zelles 1999). Because phospholipids break down rapidly in soils, the PLFA profiles derived from soil

extracts can be related directly to viable soil biomass (Frostegard *et al.* 1993; Petersen *et al.* 1991; Frostegard and Bååth 1996). Hence it was not unexpected to see the superior discriminating power of the PLFA profiles from the soil extracts compared to the FAME and NLFA profiles (Figure 3.2). The interesting differentiation between the soil under the non-mycorrhizal plants with and without added P maybe associated with large amounts of a15:0 in the soil without added P and the large amounts of 18:2ω6c in the soil with added P (Table 3.5). These fatty acids are known to be associated with bacteria and fungi respectively (Zelles 1999) and were presumably derived from saprophytic organisms, which colonised the soil during the 14 weeks of plant growth.

Table 3.7. Selected fatty acids (amounts in μg/g) from spores, root and soil samples of five treatments. (Sp = Spore, Rt = Root, Sl = Soil) obtained by FAME analysis. The fatty acids shown in this table are those relevant to the discussion. Information is compiled from Tables 3.3, 3.4 and 3.5 for clarity. Each value is an average of five replicates except for spores where values are from single samples of each species.

Fatty Acid	Source	*G. coronatum*	*G. microcarpum*	*Gi. margarita*	*Gi. rosea*	*S. calospora*
16:0 2OH	Sp	0	0	24.9	22.09	21.89
	Rt	0.88	3.33	1.58	0	0.91
	Sl	0	0	0	0	0
C16 N Alcohol	Sp	0	0	22.65	22.37	0
	Rt	0	0	0	0	0
	Sl	0	0	0	0	0
16:1ω5c	**Sp**	**40473.6**	**5652.18**	**2833.6**	**592.13**	**217.9**
	Rt	**9.10**	**47.10**	**17.4**	**1.40**	**0**
	Sl	**3.84**	**4.55**	**3.30**	**0.61**	**0.46**
i17:1	Sp	747.14	188.24	100.16	77.13	107.77
	Rt	0	0	0	0	0
	Sl	2.0	3.25	0	0	0.99
18:1ω9c	**Sp**	**2.20**	**3.15**	**305.6**	**1430**	**124.1**
	Rt	**18.1**	**14.0**	**13.2**	**19.5**	**5.2**
	Sl	**0.95**	**1.07**	**1.33**	**0.79**	**0.79**
18:2ω6c	Sp	1115	135.3	123.5	109.3	32.5
	Rt	245	331	246	162	166
	Sl	0.50	1.48	1.15	1.52	1.68
18:1ω11c	Sp	0	0	0	0	α0
	Rt	48.7	55.2	43.3	99.9	34
	Sl	1.05	2.28	2.53	2.59	2.78
20:1ω9c	**Sp**	**0**	**0**	**34.4**	**655**	**0**
	Rt	**0**	**0**	**0**	**0**	**0**
	Sl	**0**	**0**	**0**	**0**	**0**
22:1ω9c	Sp	0	0	23.1	96.3	23.6
	Rt	0	0	0	0	0
	Sl	0	0	0	0	0
C22.225	Sp	0	0	0	0	0
	Rt	0	0	2.40	15.97	0
	Sl	0	0	0	0	0
22:0	Sp	0	0	18.88	12.95	37.9
	Rt	3.60	4.98	7.53	3.76	5.40
	Sl	0	0	0	0	0

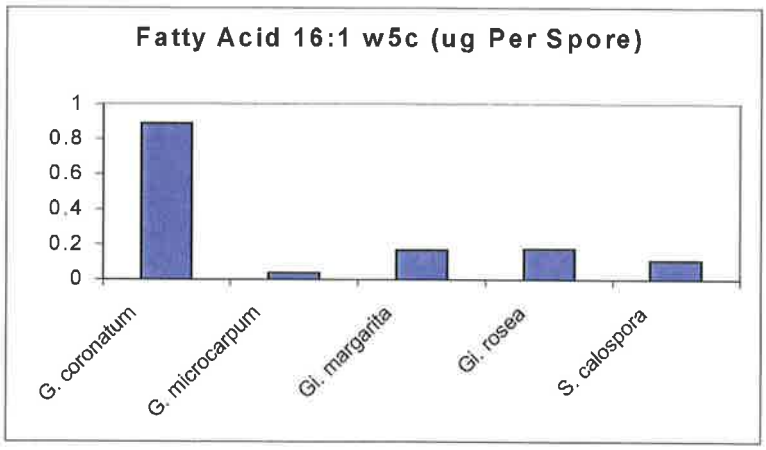


Figure 3.4 Amount of 16:1ω5c (μg) detected in the spores of five AM fungi.

Table 3.8 Comparison of the relative amount (weight %) of 16:1ω5c in the FAME, PLFA and NLFA profiles of spores, roots and soils of each treatment.

	Spores			Roots			Soils		
Treatments	FAME	PLFA	NLFA	FAME	PLFA	NLFA	FAME	PLFA	NLFA
B-P	0	N/A	N/A	0	0	0	3.54	1.25	0
B+P	0	N/A	N/A	0	0.03	0	3.75	0.97	0.04
G. coronatum	58.1	N/A	N/A	1.21	0.46	0.49	16.4	3.05	2.47
G. microcarpum	50.9	N/A	N/A	5.12	0.82	0	10.15	2.93	0
Gi. margarita	42.8	N/A	N/A	1.92	0.58	0.96	12.72	0	0
Gi. rosea	10.1	N/A	N/A	0.15	0	0.27	3.15	0	0.36
S. calospora	10.4	N/A	N/A	0	0	0	2.90	1.26	0

N/A = Not analysed

3.5.3 Evaluation of Fatty Acid Analysis in Estimation of Biomass of AM Fungal Propagules and Comparison with Morphological Data

One aim of this experiment was to check if the technique of using fatty acid analysis of roots or soils could be used to quantify the amount of AM fungal biomass present and to compare this with traditional methods of quantifying AM fungal biomass eg. % root colonisation, number of spores.

This study clearly indicated a positive correlation between the amount of 16:1ω5c detected in FAME profiles of the roots of leek and the % colonisation of roots by the different AM fungi used (Figure 3.3). Olsson *et al.* (1995) have demonstrated a positive correlation between PLFA 16:1ω5c and the length of mycorrhizal hyphae in the soil, emphasising the potential usefulness of this fatty acid for the estimation of the biomass of AM fungi in soil. However, in this study the amounts of 16:1ω5c detected in the PLFA and NLFA profiles was much smaller than that detected in the FAME profiles (Table 3.8), restricting the usefulness of the PLFA and NLFA profiles. In addition, the plant roots colonised with *S. calospora*, which were poorly colonised (14.9% ±0.2), failed to show the presence 16:1ω5c in the FAME, PLFA and NLFA profiles. This suggests that the % colonisation of roots by AM fungi may need to be greater than 15% before this fatty acid can be detected. Also, the ratio of % colonisation and the amount of 16:1ω5c detected varied with individual species suggesting that individual species should be studied to establish the relationship between % colonisation and the amount of 16:1ω5c detectable.

The amount of 16:1ω5c recovered from soil had a positive but weak correlation with the number of AM fungal spores in the soil (Figure 3.3). That this relationship was weak was not surprising considering the fact that other microorganisms present in the soil contain this fatty acid and hence contribute to a background profile. The spores of *G. coronatum* showed an unusually high amount of 16:1ω5c per spore (Figure 3.4). This suggests that the spores of this species contain larger amounts of lipid than the spores of the other species studied. Hence it may be possible to detect low levels of the spores of this species in soil samples.

3.5.4 Fatty Acid Profiles of Host Plants

The absence of AM-related fatty acids in the roots of the ten different plant species tested supports the proposal that fatty acids 16:1ω5c and 20:1ω9c can be safely used

for studying mycorrhizal fungi inside roots of host tissue. On the other hand, fatty acid 18:2ω6c, normally associated with the presence of mycorrhizal fungi in leek roots (Olsson 1999), was high (26% of the total fatty acids) in non-colonised leek roots, suggesting that this fatty acid is normally associated with roots and should not be used as a biomarker for AM fungi in plant roots.

3.6 Conclusions

In conclusion, fatty acid 16:1ω5c was confirmed as a general biomarker of AM fungi that could be detected in fatty acid profiles derived from total lipids, phospholipids or neutral lipids. As a biomarker this fatty acid could be used to differentiate between mycorrhizal and non-mycorrhizal roots, with further potential for it to be used as a quantitative biomarker for the amount of mycorrhizal colonisation of plant roots. Fatty acid 18:1ω9c could also be used as a general indicator of AM fungi and a specific indicator for species of *Gigaspora* in controlled experiments only. Fatty acid 20:1ω9c could differentiate the spores of *Gigaspora* spp. from the spores of the other AM species tested while 22:1ω9c and 22:0 could differentiate the family Gigasporaceae (including *Gigaspora* and *Scutellospora* spp) from Glomaceae at the spore level. Fatty acids 22:0 and 21:0 2OH could be used to differentiate mycorrhizal roots from non-mycorrhizal roots. Detection and quantification of the fatty acids in plant roots was slightly superior using the FAME technique (based on extraction of total lipids) compared to the lengthy PLFA / NLFA technique (based on fractionation of total lipids). The key AM-related fatty acids eg. 16:1ω5c and 20:1ω9c were not present in the non-inoculated host plants roots.

Chapter 4: Changes in the Fatty Acid Composition of Different Components of Mycorrhizal Fungi during the Development of the Plant - Mycorrhizal Association

4.1 Introduction

In Chapter 3 evidence of an association of the fatty acid 16:1ω5c with mycorrhizal structures was confirmed. However, how this fatty acid is associated with mycorrhizal development in roots over time and in what amounts, is not clear. We do not understand the distribution of 16:1ω5c in different components of mycorrhizal fungi eg. hyphae or spores and whether or not the concentrations of 16:1ω5c in colonised roots changes during the development of the symbiosis. The symbiosis between AM fungi and roots undergoes a characteristic sigmoid pattern of development shown in root colonisation. Numerous developmental changes occur to the fungus inside the host plant roots eg. formation of hyphae, arbuscules and vesicles. Therefore, it is important to examine how concentrations of 16:1ω5c in the colonised roots may change in relation to these developmental changes.

Previously, Olsson & Johansen (2000) recorded such observations for spores and hyphae of AM fungi in soil. However, there is no record of using mycorrhizal roots for similar studies. Moreover, only PLFA technique was used with fatty acids from phospholipids and neutral lipids to make generalisations. Whereas, this experiment was planned with fatty acids from total lipids, phospholipids and neutral lipids using FAME and PLFA / NLFA techniques and amounts of 16:1ω5c were followed with the development of AM fungus inside the roots and soil. Hypothetically, the amount of 16:1ω5c, like colonisation, might also be expected to follow the sigmoid pattern of development and show an initial exponential increase in the roots followed by a plateau.

Furthermore, it will be useful to establish if changes in the amounts of 16:1ω5c during the development of the symbiosis occurs uniformly in all components of the mycorrhiza or is differential, meaning, associated with only spores, hyphae or colonised roots. It was found in Chapter 3 that some fatty acids were present in spores but absent in colonised roots (intra-radical hyphae) or soils,

indicating possible differential distribution of fatty acids in mycorrhizal components. A segregation of the different components of the mycorrhizal symbiosis in an artificial growth system in a way so that the overall growth and development of the mycorrhiza is not affected, would produce a method to study changes in the amounts of 16:1ω5c and other fatty acids in mycorrhizal components as the plant mycorrhizal association develops.

The experiment discussed in this Chapter was designed with the overall objective of following the amount of 16:1ω5c in different components of the mycorrhizal association during its development.

4.2 Aims

The specific aims of this experiment were:

1. To obtain fatty acid profiles from total lipids (FAME), phospholipids (PLFA) and neutral lipids (NLFA) of segregated components of the mycorrhizal association eg. hyphae, roots colonised with the AM fungus, roots of host plants without fungi, mycorrhizal and non-mycorrhizal soils.
2. To obtain fatty acid profiles of different components of the mycorrhizal association at different time intervals in order to trace the chronology of FAME, PLFA and NLFA profiles with the development of AM fungi.
3. To compare the measurement of AM fungal biomass by fatty acid analysis with conventional methods based on counting spores and determination of % root colonisation, during development of the symbiosis.

4.3 Materials and Methods

4.3.1 Experimental Design

Glomus coronatum was selected as the experimental AM fungus and was grown on leek plants in experimental pots under glasshouse conditions for 11 weeks. The compartmented pots used in this experiment (Figure 4.1) were prepared by placing mesh bags (mesh size 33 μm) filled with 100 g of inoculum soil (soil taken from the pots with *G. coronatum* from the previous experiment) in the centre of each pot. The outer compartment of the pot was filled with 900 g autoclaved soil/sand mix (1:9 w/w Mallala soil and sand) without any fungal inoculum. The mesh was used to segregate the components of mycorrhiza, eg. soil with hyphae and spores in the outer compartment and soil with roots, hyphae

and spores in the inner compartment. Two germinated leek seeds were placed inside each soil-filled mesh bag. Ten control (non-inoculated) pots were prepared in the same manner with soil from non-mycorrhizal pot cultures placed inside the mesh bag

The pots were grown in glasshouse conditions (temperature 14-22°C, natural light) for a period of eleven weeks. All treatments received 10 ml of a modified Long Ashton fertiliser (Appendix 1) without P every week and were watered with deionised water to 10% of the weight of the soil three time a week. After 4 weeks 3 mycorrhizal pots and one control pot were harvested. This harvesting schedule was repeated every week for seven weeks. Soil from each pot was collected as follows:

- 6 g soil each from each hyphal chamber and root chamber for FAME analysis
- 12 g soil each from hyphal chamber and root chamber for PLFA / NLFA analysis
- 50 g soil each from each hyphal chamber and root chamber for spore counting

Roots of the host plants from the root chamber were harvested and washed before a random 0.15 g sample was collected for measurement of root colonisation. From the control pots, the soil was collected only from the root chamber for FAME, PLFA / NLFA analyses and spore counts.

4.3.2 Morphological Observations

The spores were extracted by wet sieving (Chapter 2, section 2.8) and counts were made on 3 replicate mycorrhizal samples and one non-inoculated control from each of hyphal chamber (HC) and root chamber (RC). The roots were stained and examined for % colonisation as described in section 2.7 of Chapter 2.

4.3.3 FAME and PLFA / NLFA Analysis

FAME, PLFA and NLFA profiles of roots and soil samples were obtained using the protocol described in sections 2.9 and 2.10 of Chapter 2.

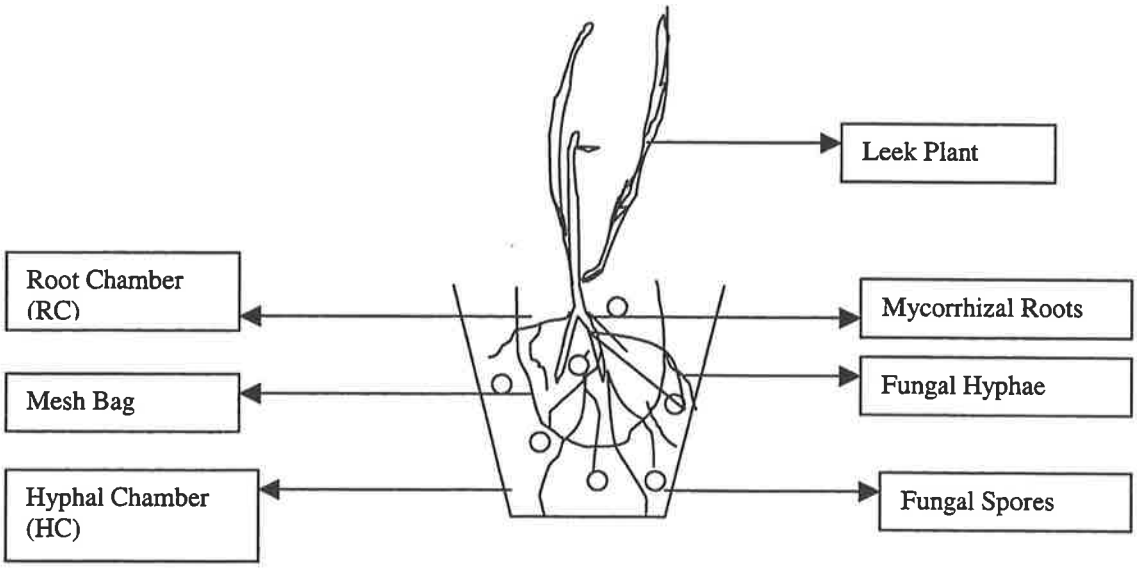


Figure 4.1: Experimental pot design where a mesh bag (33 μm) separates the root chamber from the hyphal chamber.

4.3.4 Statistical Analysis of Data

Simple linear regression and analysis of variance (ANOVA) were used to analyse the data. The objective of applying these techniques to the FAME data from roots and soils was to determine whether the amounts of 16:1ω5c in the RC differed from that in the HC over time. A regression approach assumes that the data is normally distributed with constant variance. To satisfy this assumption the data for 16:1ω5c was log transformed.

For soil PLFA / NLFA data, analysis of variance was used to analyse data assuming a 2x2x7 factorial treatment structure [referring to soil from 2 chambers, with 2 types of fatty acids over 7 harvests]. The data was transformed on the log scale to satisfy assumptions of analysis of variance. It also may be noted that one observation (Rep 1, of phospholipid in hyphal chamber, in Week 2 of harvest) had a very high value, which was attributed to an error in the data hence was omitted.

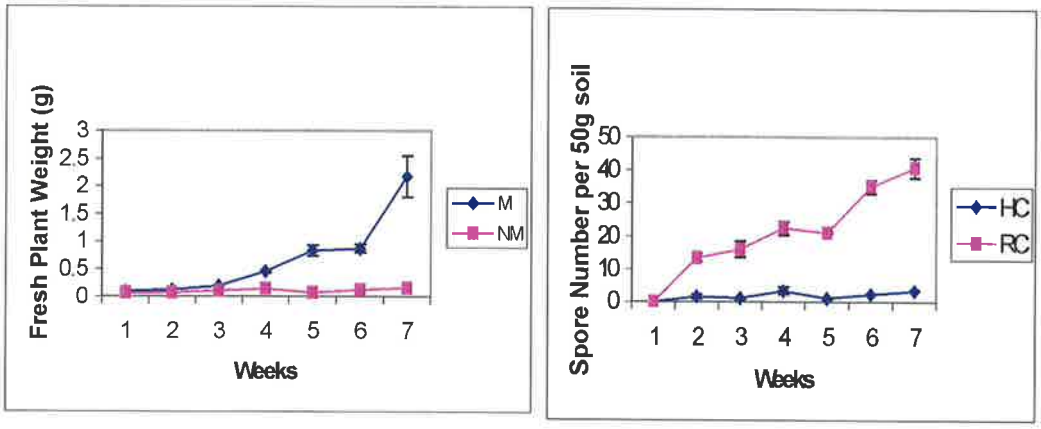
4.4 Results

4.4.1 Growth and Morphological Observations

The fresh weight of mycorrhizal plants increased steadily over the 7-week harvest period but growth of non-mycorrhizal plants was negligible (Figure 4.2a). The number of spores in the HC showed an increase over the 7-week period from none in the first week to 3.3 spores/50 g soil in the 7^{th} week of the harvest (Figure 4.2b). A similar trend was observed in the root chamber where the number of spores increased from none to 40.6 spores/50 g soil in the 7^{th} week.

Due to insufficient root material during the initial weeks of the experiment, the roots were checked for % colonisation for harvest weeks 5, 6 and 7 only (Figure 4.2c). All material harvested of four weeks was used for FAME analysis. Average % root colonisation was highest in harvest week 5 and it showed a slight, but non-significant decline, in harvest weeks 6 and 7 (Figure 4.2c).

(a) (b)



(c) (d)

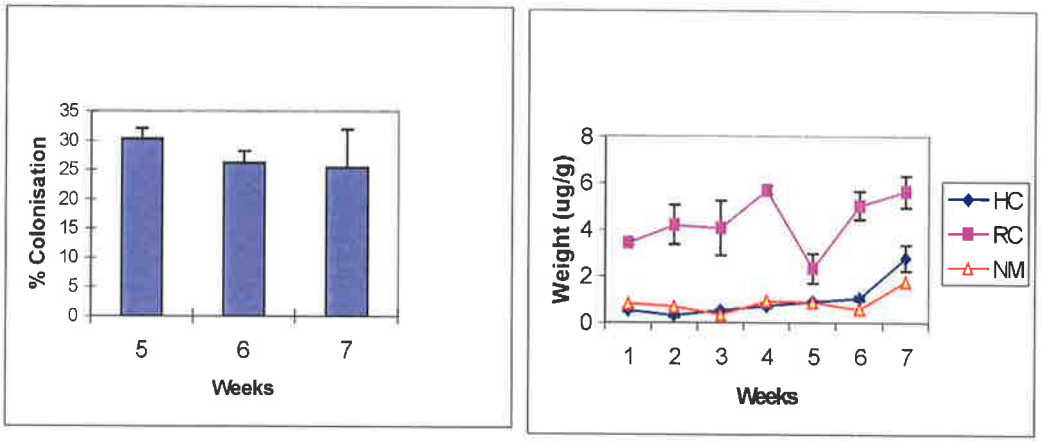


Figure 4.2a Average increases in the fresh weight of mycorrhizal (n=3) and non-mycorrhizal plants (n=1) over a 7-week harvest period.

Figure 4.2b Average increase in the number of spores in the HC and RC from week 1 to week 7 of the harvest (n=3).

Figure 4.2c Percent root colonisation in the roots of host plants from harvest week 5 to harvest week 7.

Figure 4.2d Amount (μg/g) of 16:1ω5c detected in FAME profiles of soils in the HC and RC of mycorrhizal plants (n=3) and non-mycorrhizal plants (n=1). [HC = hyphal chamber, RC = root chamber NM = non-mycorrhizal, M = mycorrhizal]

4.4.2 FAME and PLFA / NLFA Analysis

4.4.2.1 Soils

The concentration of fatty acid 16:1ω5c detected in the FAME, PLFA and NLFA profiles of soils from the HC and RC of mycorrhizal pots and the RC of non-mycorrhizal pots is shown in Table 4.1. The results showed that 16:1ω5c increased from a low initial concentration in both chambers of mycorrhizal pots, while the values from non-mycorrhizal pots were patchy and there was no clear trend between harvest weeks 1 and 7. By FAME analysis, the concentration of 16:1ω5c (μg/g) was six times higher in the RC than the HC at harvest week 1 (Table 4.1, Figure 4.2d) and this degree of difference between the RC and the HC continued until the last harvest (harvest week 7) except for low values in week 5. This difference in concentration of 16:1ω5c was also consistent with the difference in spore density (spores / 50 g soil) between the RC and HC (Figure 4.2b). The concentration of 16:1ω5c detected from the PLFA profiles was less consistent with a very high value obtained at harvest week 2 and a low value obtained at harvest week 5 in the RC. However, it was of interest to note that the degree of difference in the amount of 16:1ω5c detected between the RC and the HC was less with the PLFA analysis than with the FAME analysis. From harvest week 5 to 7 the amount of 16:1ω5c detected by PLFA was higher in the HC than the RC (Table 4.1). Fatty acid 16:1ω5c was not detected in the NLFA profiles of the RC and on only two occasions in the HC (Table 4.1).

Statistical analysis of the FAME data revealed that the amount of 16:1ω5c increased at a similar rate in both the RC and HC. However, there was significantly more 16:1ω5c in the RC compared to the HC regardless of the harvest time (Figure 4.3). Analysis of variance of the PLFA data revealed no interaction between chamber type and harvest week at the 5% significance level.

Table 4.1. Amounts of 16:1 ω5c (μg/g soil) in FAME, PLFA and NLFA profiles of soils from the hyphal chamber (HC) and root chamber (RC) of the mycorrhizal leek plants and the RC of non-mycorrhizal leek plants (NM) over a period of seven weeks. All HC and RC values are the mean of three replicates and NM values are from single pots. The values in parenthesis are standard errors.

Harvest week		**1**	**2**	**3**	**4**	**5**	**6**	**7**
FAME	**NM (RC)**	0.81	0.68	0.34	0.94	0.89	0.58	1.77
	HC	0.54 (0.08)	0.31 (0.09)	0.52 (0.05)	0.71 (0.07)	0.90 (0.06)	1.05 (0.11)	2.78 (0.57)
	RC	3.44 (0.12)	4.20 (0.55)	4.06 (0.18)	5.69 (0.21)	2.33 (0.65)	5.03 (0.62)	5.64 (0.69)
PLFA	**NM (RC)**	13.46	0.04	0	2.67	3.54	2.17	0
	HC	1.63 (0.16)	4.97 (1.43)	7.99 (1.29)	4.78 (0.64)	2.22 (0.72)	7.02 (0.75)	1.02 (0.04)
	RC	2.78 (0.12)	24.18 (5.07)	5.59 (0.31)	5.34 (0.44)	0.95 (0.04)	3.38 (0.07)	0.47 (0.01)
NLFA	**NM (RC)**	2.28	0	3.53	0	0	0	0
	HC	2.08 (0.45)	0	0	1.86 (1.07)	0	0	0
	RC	0	0	0	0	0	0	0

Source of variation	**v.r.**	**F. Pr.**
Chamber	0.02	0.883
Type	6.88	0.011
Week	12.27	<0.001
Chamber x Type	0.04	0.844
Chamber x Week	1.81	0.113
Type x Week	3.17	0.010
Chamber x Type x Week	1.31	0.269

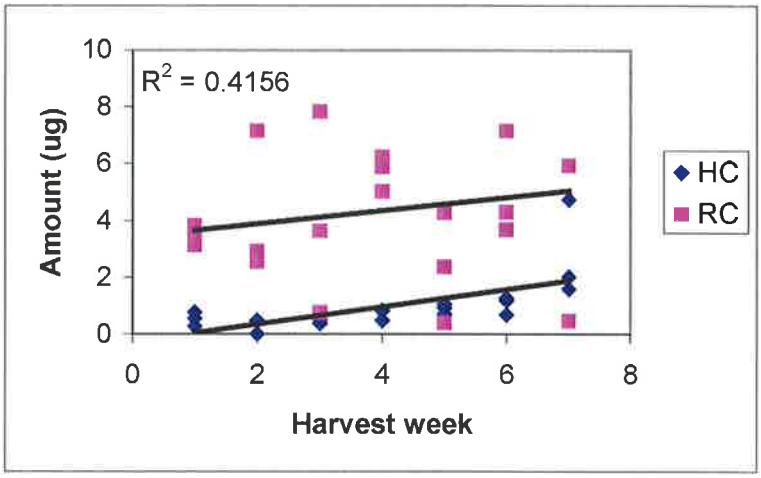


Figure 4.3 Relationship between time of harvest (weeks) and the amount of 16:1ω5c (μg) detected in FAME profiles of soil in the HC and RC.

4.4.2.2 Roots

Due to insufficient root material at the first three harvests, FAME, PLFA and NLFA profiles of the roots of colonised and non-colonised host plants were obtained at three harvest times (weeks 5-7) only. Fatty acid 16:1ω5c was detected in the FAME and PLFA profiles of the mycorrhizal roots but not the non-mycorrhizal roots. The amounts detected varied, and it was not detected in some replicates. However, the amount detected appeared to peak around harvest weeks 5 and 6 with the greatest amount detected in the FAME profiles of mycorrhizal roots at week 6 (Table 4.2). Fatty acid 16:1ω5c was not detected in the NLFA profiles of mycorrhizal or non-mycorrhizal roots.

Table 4.2 Amount of 16:1ω5c (μg/g roots) in FAME, PLFA and NLFA profiles of mycorrhizal (M) and non-mycorrhizal (NM) plant roots.

Harvest week		**4**	**5**	**6**	**7**
FAME	M	7.7	74.1	107.4	0
	NM	0	0	0	0
PLFA	M	0	22.2	0.96	3.9
	NM	0	0	0	0
NLFA	M	0	0	0	0
	NM	0	0	0	0

4.5 Discussion

4.5.1 Morphological Observations

Mycorrhizal plants grew faster than the non-mycorrhizal plants and had 5 times greater fresh weight at the end of the experiment (Figure 4.2a). This increase in growth was consistent with the mycorrhizal colonisation of the plant roots, which had reached 30% of total root length by 9 weeks of growth (5^{th} harvest) and remained relatively constant until the end of the experiment. Spore density in the RC also increased between harvest weeks 2 and 7 and there appeared to be two phases of spore production; the first between harvest weeks 1 and 3, the second between harvest weeks 5 and 7 (Figure 4.2b). Spore density in the HC remained low throughout the experiment, and showed only a small increase during the 7-week harvest period. The low density of spores in the HC may have been due to failure of hyphae to colonise the HC or to a lower efficiency of the hyphae to produce spores the further it was from the colonised plant roots.

4.5.2 FAME Analysis of Soils and Roots

The experiment was designed to test the hypothesis that the increase in plant weight and root colonisation by AM fungi during the test period would be reflected in the amounts of fatty acid 16:1ω5c detected in plant roots, as shown in Chapter 3 and by other authors (Graham *et al.* 1995; Olsson *et al.* 1995). In this experiment, the effect of mycorrhizal colonisation of the rhizosphere and plant roots on the amount of 16:1ω5c present was tested by extracting soil samples from the root chamber (RC) (where roots, hyphae and spores were present) and from the hyphal chamber (HC) (where only hyphae and spores were present). Consistent with the morphological observations, there was an increase in the amount of 16:1ω5c in the FAME profiles from both soil chambers (Figure 4.3), although values were significantly higher (up to 13 times at harvest week 2) in the RC compared to the HC. This indicated a marked difference in the amount of fungal biomass between the RC and the HC. Physical evidence for this difference in biomass could be seen from the spore counts (Figure 4.2b) which showed the progressive development of large numbers of spores in the RC but not in the HC. However, if the actual amount of 16:1ω5c that can be attributed to spores is calculated using a figure of 0.48 μg of 16:1ω5c per spore (determined for *G. coronatum* in Chapter 5), it was found that the spores contributed very little to

amount of 16:1ω5c detected in the RC. At harvest week 4, there were approximately 20 spores per 50 g of soil (or 0.4 spore per g soil) in the RC. The amount of 16:1ω5c theoretically contributed by the spores at harvest week 4 was therefore 0.2 μg / g soil, compared to the 5.7 μg / g soil detected (Table 4.1). Thus it can be assumed that the bulk of the 16:1ω5c detected in the RC at each harvest week was largely contributed by other AM fungal structures (hyphae, vesicles etc.). This being the case, it can be assumed that the amounts of 16:1ω5c detected in the RC during harvest weeks 1 to 4 (where root colonisation could not be measured due to insufficient root material) was associated with growth and development of the AM fungus in the soil, rhizosphere and roots. However a sudden drop in the amount of 16:1ω5c in the harvest week 5 appeared to be due to collective loss of spores and hyphae in this week as was observed in the spore counts (Figure 4.2b). This drop in the amount of mycorrhiza could not be explained other than the presence of less hyphae, as there was no apparent change in any physical or growth condition. But the fact that the change in physical amounts of mycorrhizas was reflected in the amounts of fatty acid 16:1ω5c, further substantiated the correlation of the two.

4.5.3 PLFA / NLFA Analysis of Soils and Roots

Phospholipids have been used as an indicator of viable microbial biomass in the soil (Frostegard *et al.* 1997). This is possible because they are (1) an essential component of living cell membranes and (2) they breakdown rapidly in the soil following the death of cells (White *et al.* 1979). In AM fungi, a large proportion of 16:1ω5c comes from phospholipids (Beilby 1980; Nordby *et al.* 1981; Pacovsky & Fuller 1988) and this fatty acid is not commonly found in other fungi (Müller *et al.* 1994). Hence the amount of 16:1ω5c detected in PLFA profiles of soil is considered a relative measure of the biomass of living AM fungi in soil (Olsson *et al.* 1995; 1997; Olsson 1999).

In this experiment, the greatest amount of 16:1ω5c detected in PLFA profiles of the soil in both the RC and HC occurred between harvest weeks 2 and 4. The amount detected in the RC was similar to that detected in the RC with the FAME analysis. As the bulk of the 16:1ω5c detected by FAME in the RC was associated with AM fungal structures other than spores (discussed above), the

similarity in the amount of 16:1ω5c detected by the FAME and PLFA analysis in the RC was not surprising as one would assume that most of the fatty acid detected in the PLFA analysis would be derived from membranes in hyphae and vesicles. In contrast, the amount of 16:1ω5c detected in the PLFA profiles of the soil from the HC was much greater than that detected in the FAME profiles of soil from the HC. The reason for this is unclear, and may reflect a difference in the capacity of the two techniques to detect 16:1ω5c in soil samples, especially if it is largely associated with viable fungal hyphae. The peak in the amount of 16:1ω5c detected in the PLFA profiles between harvest weeks 2 and 4 could coincide with the period of active colonisation of the host plant roots by the AM fungus. In absence of the data for root colonisation during the first 3 harvest weeks, however, it cannot be determined whether the colonisation was increasing during this period, or remained steady.

There may have been some technical problems associated with the inconsistent detection of 16:1ω5c in the FAME and PLFA profiles of the mycorrhizal roots. It was anticipated (see Figure 3.3, Chapter 3) that the amount of 16:1ω5c would show an increase as the % colonisation of the roots increased. Whilst this did occur with the amount of 16:1ω5c detected in the FAME profiles between harvest weeks 4 and 7, the same increase did not occur with the PLFA profiles. This could be associated with the small root samples used (0.3 g) in the assays and non-uniform colonisation of roots by the AM fungus. Also, since the roots were stored at –4°C for several weeks, it was possible that there was some breakdown of the lipids in the samples before analysis. However, despite these possible technical problems, the data did show the presence of detectable 16:1ω5c in the roots at the time of maximum root colonisation by the AM fungus.

The failure to detect 16:1ω5c in the NLFA profiles of the roots could be due to the fact that neutral lipids are unlikely to be present in root cells because they are associated more with storage structures eg. spores (Tunlid & White 1990).

4.6 Conclusion

The following conclusions could be drawn from this experiment.

- There was a steady increase in the growth of plants inoculated with *G. coronatum* through the test period of 7 weeks. Mycorrhizal development

during this period was confirmed by an increase in the number of spores around the plant roots and by root colonisation.

- The concentration of fatty acid 16:1ω5c detected by FAME or PLFA analysis of the soil associated with the plant roots increased initially then remained either stable or decreased, showing a comparable trend to the sigmoid growth of mycorrhiza formation.
- The amount of 16:1ω5c increased in both the chambers (RC, HC) at the same rate irrespective of the harvest time, but the RC had significantly higher amounts of 16:1ω5c.
- The majority of the 16:1ω5c detected in the RC was not associated with AM fungal spores but rather with hyphae or other mycorrhizal structures.

Chapter 5: FAME Analysis of Spores of AM Fungi and Applicability of Fatty Acids Analysis at Species Level

5.1 Introduction

Results of the first experiment of applying the FAME and PLFA techniques to five AM species (Chapter 3) clearly indicated the presence of large quantities of 16:1ω5c, 18:1ω9c and some 20-C fatty acids in AM fungal spores. In the time course experiment (Chapter 4) however, the FAME analysis was found slightly better than the PLFA analysis in providing a quantitative correlation of the amounts of 16:1ω5c with mycorrhiza development by *Glomus coronatum*. In that experiment, only one AM species was used, which restricted the application and inference of the results to other AM species. In order to understand the quantitative relationship between the fatty acids and mycorrhizas, a different approach was necessary. Since spores were considered the only 'uncontaminated' source of AM fungi, an alternative approach of using FAME analysis of only the spores was chosen in this Chapter. The other components of mycorrhizas are encountered in a symbiotic state eg. hyphae are present either with the roots or with the spores in soil. The spores are the only components of mycorrhiza capable of showing a direct link with the presence or absence of fatty acids and the hypothesis was that the quantities of AM-related fatty acids should increase with the increasing number of spores of a species.

A major objective of the work described in this Chapter was to confirm the FAME profiles of the spores of AM fungi examined previously (Chapter 3) and to study the relationship of specific fatty acids to spores of particular AM species (species described in section 5.3.2), including a new AM species (*Glomus mosseae*). *G. mosseae* species was substituted for previously used *G. microcarpum* (Chapter 3) because the larger size of its spores allowed the spore numbers to be counted more accurately. Due to smaller size of spores of *G. microcarpum* the spores could only be estimated and not counted precisely. Another objective was to determine whether the fatty acids in a known number of spores could be quantitatively recovered when the spores were deliberately added to a soil-sand mix. Adding a known number of spores would allow the predicted amounts of fatty acids

to be controlled. This approach overcomes problems related to the relative unpredictability of mycorrhiza developed in pots (Chapter 4). The possibility that amounts of fatty acids extracted from soil could be used to estimate the number of AM spores in soil, was examined. The effect of crushing spores on the yield of fatty acids was also determined.

In Chapter 3, useful information about the specific linkage of fatty acids with AM species was observed. For example some 20-C fatty acids eg. 20:1ω9c, 20:2ω6c, 20:3ω6c, 20:4ω6c, 22:1ω9c and 24:1ω9c appeared to be selectively associated with species of *Gigaspora* or members of the family Gigasporaceae. Furthermore, 18:1ω9c was detected in comparatively higher amounts in spores of *Gi. margarita* than other species. The work described here was aimed at confirming this observation and extending observations to other members of the genus *Gigaspora*. No previous studies have determined the usefulness of fatty acid analysis for the identification of AM fungi at isolate or species levels. The Microbial Identification System (Microbial ID, Inc., Newark) is successfully used extensively for bacterial strain identification using fatty acids as tools (Sasser 1990) and could potentially be useful for AM fungi if the specific fatty acids could be linked to specific species of AM fungi.

5.2 Aims

The main aims of this experiment were:

1. To establish a quantitative relationship between the number of spores of selected AM fungi with the amounts of AM-related fatty acids identified in previous experiments (Chapter 3).
2. To obtain FAME profiles of a known number of spores in a soil background.
3. To obtain FAME profiles of different combinations of spores of 3 AM species in a soil background.
4. To determine the effect of mild crushing on spore FAME profiles.
5. To determine if there was a correlation between the concentration of 16:1ω5c per spore and the weight of a spore.

6. To verify whether the potential of diagnostic fatty acids for *Gigaspora* existed at genus or species level.

5.3 Materials and Methods

5.3.1 Experimental Design

The following experiments were performed to achieve the objectives:

1. FAME analysis of groups of different number of spores.
2. FAME analysis of 100 spores of each AM species.
3. Effect of crushing spores on the yield of fatty acids.
4. FAME analysis of a mixture of spores with soil.
5. Weight of spores compared with concentration of 16:1ω5c in AM species.
6. Testing FAME at species level with *Gigaspora* species.

5.3.2 Fungal Isolates and Spore Collection

The AM fungi used were *Glomus coronatum*, *G. mosseae*, *Gigaspora margarita* and *Scutellospora calospora*. Five replicate pots with each species were grown on the host plant (leek) for 14 weeks in a glasshouse as described previously (Chapter 2). For fatty acids profiles of species of *Gigaspora* (*Gi. margarita, Gi. rosea* and *Gi. decipiens*) 10 pots with each species were grown for 12 weeks in glasshouse conditions. The soil from pots in which these species were grown previously, was used as inoculum and a sample of 100 g soil was mixed with 1300 g of 10% Kuitpo soil in each pot. The plants were measured for their fresh shoot and root weights to assess the plant growth after 12 weeks. The soil from each pot was sampled for FAME analysis (6 g) and for spore isolation (100 g). The roots of the host plants were collected on a sieve, washed thoroughly to remove soil and sampled for % colonisation estimation (0.15 g) and FAME analysis (0.3 g). Spores were extracted from the soils using the wet sieving technique (see 2.8). The host roots were observed for percent of the total root length colonised after the roots were cleared in KOH and stained, using the line intersect method (see 2.7). FAMEs were extracted from soil, root and spore samples. For FAME extraction from spores, 5 replicate

samples of 100 spores were used. For FAME extraction from roots, 10 replicates of a 0.3 g root sample and for soils 10 replicates of a 6 g sample were taken. The procedure has been described in detail in section 2.9 of Chapter 2.

5.3.3 Treatment of Spores

The weight of spores of each AM fungus was determined as follows:
Three replicate samples of 100 spores of each of the four species [*Glomus coronatum* (GC), *G. mosseae* (GM), *Gigaspora margarita* (MARG) and *Scutellospora calospora* (SC)] were deposited in pre-weighed glass tubes in a drop of water. The water was then evaporated using a jet of compressed air and the tube reweighed. The results were used to calculate the mean weight per spore, and hence determine the weights of known numbers of spores added to soil. In the spore-series experiment, the numbers of spores used for each species were 25, 50, 100 and 200 (Figure 5.1).

In an experiment to test the stability of the spore FAME profiles in a background of soil, batches of 100 spores of either single or mixed species of three AM fungi [*G. mosseae* (GM) *Gi. margarita* (MARG) and *S. calospora* (SC)] were added to 6 g sand-soil mix, and the fatty acids extracted using the method as described earlier. The number of spores used in this experiment were: GM, 100; MARG, 100; SC, 100; GM, 75 + MARG, 25; GM, 25 + MARG, 75; GM, 75 + SC, 25; GM, 25 + SC, 75.

The effect of crushing spores on the extraction of lipids was tested by comparing the FAME profiles of 3 replicate samples of crushed and uncrushed spores (100 spores per sample) of three AM species. Crushing was carried out in a 2ml Eppendorf tube with a plastic plunger in 50 µl of 3.75M NaOH in 50% aqueous methanol to produce a slurry, which was then processed for extraction of fatty acids.

5.3.4 FAME Analysis of Roots, Soils and Spores

Fatty acids were extracted using the protocol as described in section 2.9 of Chapter 2. For the three species of *Gigaspora*, the inoculum soil was also analysed using FAME analysis, in order to assess the initial amounts of fatty acids in the inoculum.

5.4 Results

5.4.1 Spore Analysis

5.4.1 1 FAME Analysis of 100 Spores

The amounts of different fatty acids extracted from 100 spores of four AM species are shown in Table 5.1. Fourteen fatty acids were routinely detected with large amounts of fatty acids 16:0 and 16:1ω5c present in the spores of all species. *Gi. margarita* contained very large amounts of 18:1ω9c compared with the 2 *Glomus* species and *S. calospora* and also contained significant quantities of 3 fatty acids, 20:1ω9c, 20:2ω6c and 22:1ω9c, which were not detected in the other 3 AM species. All other 18-C and 20-C fatty acids detected were significantly higher in *Gi. margarita* than the other species suggesting that it might be possible to detect spores of *Gi. margarita* against a background of spores of the other three AM species.

5.4.1.2 Amounts of 16:1ω5c Extracted from Increasing Number of Spores

An analysis of 25, 50, 100 and 200 spores each of four AM species showed that amounts of 16:1ω5c increased with the increasing number of spores in all species (Figure 5.1). This increase, although roughly linear, was less apparent with the spores of *G. coronatum* and *S. calospora* as compared to the spores of *Gi. margarita* and *G. mosseae*. The highest amounts of 16:1ω5c were observed in spores of *G. coronatum* and least in spores of *G. mosseae* (Figure 5.1).

5.4.1.3 Effect of Crushing Spores on Recovery of FAMEs

Crushing spores prior to lipid extraction caused a decrease in the yield of fatty acids detected in all three AM fungi tested (Table 5.2). However, the decrease in yield was only significant with fatty acid 16:1ω5c.

5.4.1.4 FAME Analysis of Spores added to Soil

The amount of the fatty acids extracted from soil containing different combinations of spores of the 3 AM fungi tested is shown in Table 5.3. With the exception of one spore combination (25 SC + 75 GM spores) which yielded less total fatty acids than

the control soil (no spores added), the amounts of the 14 fatty acids detected across the 3 AM species tested were greater in the spore + soil preparations than in the soil alone. Fatty acids 20:1ω9c, 20:2ω6c, 20:4ω6c and 22:1ω9c were only detected when *Gi. margarita* was present. The amounts of fatty acids detected in the spore combinations were approximately proportional to the number of spores added to the soils. For example the amount of fatty acid 20:1ω9c, found only in *Gi. margarita* spores, was detected in combinations of 25 MARG + 75 GM and 75 MARG + 25 GM at 34 and 72% of the amount for 100 MARG spores (Table 5.3).

5.4.1.5 Spore Weight versus Amount of 16:1ω5c

The average weight per spore of four AM fungi is shown in Figure 5.2a. The spores of *S. calospora* were the heaviest and the spores of *G. coronatum* were the lightest among the 4 species tested. The weight of spores however did not correspond with the amount of fatty acid 16:1ω5c per spore of these species (Figure 5.2b). The lighter spores of *G. coronatum* had the highest amount of 16:1ω5c per spore.

Table 5.1 Amounts (μg) of different fatty acids detected in the FAME profiles of 100 uncrushed spores of four AM species. Each value represents a mean of 3 replicate samples for each species.

Fatty Acids	*G. coronatum*	*G. mosseae*	*Gi. margarita*	*S. calospora*	LSD (P<0.05)
16:0	13.1	25.2	133.6	20.3	48.4
16:1 ω5c	48.5	44.2	22.7	33.8	17.5
18:1 ω5c	0.23	0.04	7.02	0	2.0
18:1 ω7c	9.8	8.7	27.6	5.8	10.4
18:1 ω9c	1.7	3.5	136.8	3.9	49.3
18:2 ω6c	0.06	0.5	4.0	0.5	1.0
18:3 ω6c	0.5	0.6	3.07	0.3	0.9
20:1 ω9c	0	0	31.1	0	5.3
20:2 ω6c	0	0	11.2	0	3.9
20:3 ω6c	0	0.3	8.2	0	3.1
20:4 ω6c	0	0.3	6.1	0	2.5
20:5 ω3c	0	0.3	6.2	0.07	2.3
22:1 ω9c	0	0	5.2	0	1.1
24:1 ω9c	0.5	1.3	3.6	0.06	2.3
Total	77.7	90	414.7	67.5	

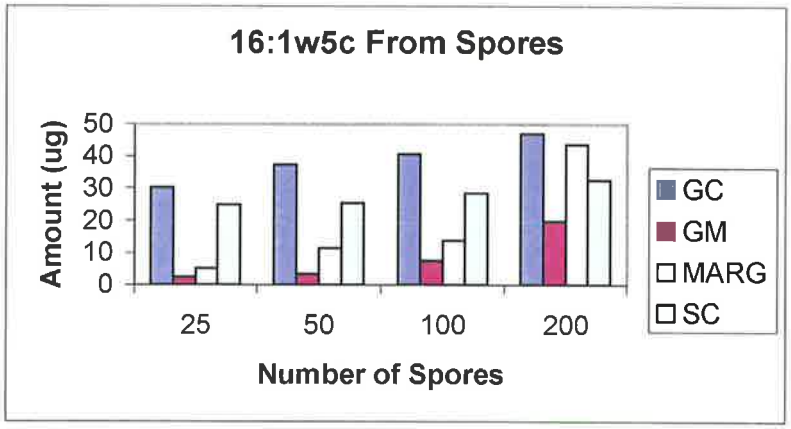


Figure 5.1 Amount (μg) of 16:1ω5c detected in varying number of spores of four AM species. Each value represents a single sample. [GC = *Glomus coronatum*, GM = *G. mosseae*, MARG = *Gigaspora margarita*, SC = *Scutellospora calospora*]

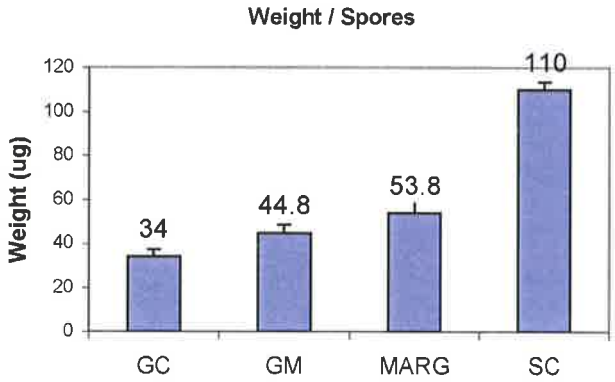
Table 5.2 Amounts (μg) of fatty acids detected in the FAME profiles of crushed (C) and uncrushed (UC) spores of three AM species. Each value is a mean of three replicate samples.

Table 5.2 Amounts (μg) of fatty acids detected in the FAME profiles of crushed (C) and uncrushed (UC) spores of three AM species. Each value is a mean of three replicate samples.

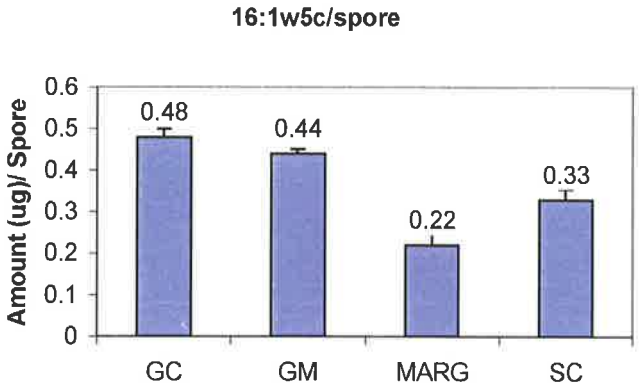
Fatty Acids	*Scutellospora calospora*		*Gigaspora margarita*		*Glomus mosseae*		LSD (P<0.05)	
	C	UC	C	UC	C	UC	Treat	Sp x Treat
16:0	22	29	80	188	14	33	0.172	0.393
16:1 ω5c	37.01	54.9	13.9	31.6	22.3	54.2	0.011	0.689
18:1 ω5c	0	0	3.24	8.84	0	0	0.224	0.291
18:1 ω9c	3	4	81	193	3	6	0.224	0.325
18:2 ω6c	0	0.67	1.72	4.11	0	0.86	0.222	0.754
18:3 ω6c	0	0.78	0.75	3.19	0	0	0.187	0.526
20:1 ω9c	0	0	22.8	53.7	0	0	0.206	0.264
20:2 ω6c	0	0	69	15.5	0	0	0.260	0.344
20:3 ω6c	0	0.6	5.0	11.4	0	0	0.257	0.428
20:4 ω6c	0	0	2.99	8.46	0	0	0.288	0.386
20:5 ω3c	0	0.51	2.60	7.83	0	0	0.269	0.459
22:1 ω9c	0	0	5.66	6.44	0	0	0.769	0.928
24:1 ω9c	0	0.30	1.37	2.12	0	0	0.474	0.856
C25 N Alcohol	0	0.41	0.15	0	0	0	0.104	0.015

Table 5.3. Amounts (μg) of fatty acids detected in FAME profiles of soil containing 100 AM fungal spores, either of one species or mixed spores of two species. [GM = *G. mosseae*, MARG = *Gigaspora margarita*, SC = *Scutellospora calospora*]. Each value is the mean of three replicate samples.

Fatty Acid	Control Soil	100 GM	100 MARG	100 SC	25 MARG +75 GM	75 MARG +25 GM	25 SC +75 GM	75 SC +25 GM	LSD P<0.05
16:0	37.7	51.0	189.2	84.3	91.2	177.6	43.1	73.6	29.5
16:1 ω5c	8.4	31.5	33.3	85.3	31.8	43.2	39.7	69.9	19.5
18:1 ω5c	0	0.9	7.9	0.5	3.1	7.2	0	0.8	1.3
18:1 ω7c	4.9	10.5	29.3	18.7	16.2	28.1	9.6	16.1	5.3
18:1 ω9c	22.4	27.7	208.1	29.6	82.8	180.4	17.9	27.0	25.3
18:2 ω6c	12.2	12.0	13.1	9.1	7.8	11.1	4.2	11.9	5.2
18:3 ω6c	1.2	2.3	2.3	1.4	2.2	2.6	1.1	1.3	0.7
20:1 ω9c	0	0	57.5	0	19.2	41.2	0	0.9	8.8
20:2 ω6c	0	0	17.7	0	5.8	14.1	0	0	2.3
20:3 ω6c	0	1.2	6.6	1.2	3.2	6.1	0.4	1.1	1.3
20:4 ω6c	0	0	4.7	0	2.1	4.4	0	0	0.7
20:5 ω3c	0	0.5	4.9	1.4	2.4	5.2	0.3	1.2	1.1
22:1 ω9c	0	0	6.7	0	3.2	3.7	0	0	2.3
24:1 ω9c	0	0	1.8	0.2	1.4	0.6	0	0.4	0.8
Total	572	757	1490	857	1012	1516	523	759	



(a)



(b)

Figure 5.2 (a) Average weight per spore and (b) average amounts of fatty acid 16:1ω5c per spore from 4 AM species. [GC = *Glomus. coronatum*, GM = *G. mosseae*, MARG = *Gigaspora margarita*, SC = *Scutellospora calospora*]. The numbers on top of the bars are (a) weight (μg) per spore and (b) amount (μg) of 16:1ω5c per spore of four AM species.

5.4.2 FAME Analysis of *Gigaspora* Species

5.4.2.1 Plant Growth and Mycorrhizal Colonisation

Plant growth stimulation, production of spores and % root colonisation, by the test *Gigaspora* species at the end of the 12 weeks growth period, is shown in Table 5.4. Leek plants colonised by *Gi. margarita* showed the best growth followed by those colonised by *Gi. rosea*. Both of these species produced appreciable numbers of spores in the soil surrounding the roots and both showed also good colonisation of the plant roots. In contrast, plants inoculated with *Gi. decipiens* showed growth similar to non-colonised plants and % colonisation observations confirmed that less than 4% of the total root length was colonised with this species. Very few spores were found in the soils under plants inoculated with *Gi. decipiens*. Lack of spore production meant that no further analysis could be performed on spores of *Gi. decipiens*.

Table 5.4 Plant growth, production of spores and % of total root length colonised by 3 species of *Gigaspora*. Each value represents a mean of 10 replicates ± the standard error.

Treatments	Shoot Weight (g)	Root Weight (g)	Number of Spores/g soil	% Root Colonisation
Control	0.208 ±0.005	0.140 ±0.004	0.108 ±0.009	0
Gi. margarita	1.568 ±0.038	0.821 ±0.01	2.54 ±0.156	58.5 ±0.85
Gi. rosea	0.442 ±0.01	0.257 ±0.007	1.22 ±0.06	44.2 ±0.83
Gi. decipiens	0.219 ±0.003	0.145 ±0.002	0.02 ±0.003	3.8 ±0.34

Table 5.5 Amounts (μg) of fatty acids detected in FAME profiles of 100 spores collected from soil (see section 5.3.2) each of *Gi. margarita* and *Gi. rosea*. Each value is a mean of five replicates. Critically discussed fatty acids are shown in bold in this table.

Fatty Acids	*Gi margarita*	*Gi rosea*	LSD	Significance (P<0.05)
16:0	316.8	63.6	53.6	<0.001
16:1ω5c	**67.1**	**4.5**	**11.2**	**<0.001**
16:1ω9c	1.07	0	0.81	0.022
18:1ω7c	62.9	16.2	10.1	<0.001
18:1ω9c	**387**	**118**	**59.9**	**<0.001**
18:2ω6c	13.1	4.01	2.75	<0.001
18:3ω6c	2.24	0	0.67	<0.001
20:1ω9c	**116.4**	**54.6**	**14.3**	**<0.001**
20:2ω6c	37.0	11.8	6.57	<0.001
20:3ω6c	10.3	3.90	1.80	<0.001
20:4ω6c	6.91	1.97	1.51	<0.001
20:5ω3c	7.78	3.53	2.34	0.007
22:1ω9c	**13.45**	**8.59**	**2.68**	**0.007**
24:1ω9c	4.23	1.51	1.93	0.018

5.4.2.2 FAME Analysis of Spores

Average amounts of fatty acids (μg) detected in FAME profiles of 100 spores each of *Gi. margarita* and *Gi. rosea* are shown in Table 5.5. Fatty acids 16:0, 18:1ω7c, 18:1ω9c, 20:1ω9c, 20:2ω6c and 22:1ω9c were found to be dominant in both species although the amounts were significantly lower in *Gi. rosea* as compared to *Gi. margarita*. Fatty acid 16:1ω5c was much higher in *Gi. margarita* than in *Gi. rosea*.

5.4.2.3 FAME Analysis of Roots

Average amounts of fatty acids (μg/g root) detected in FAME profiles of the roots of leek plants colonised by *Gi. margarita* and *Gi. rosea* are shown in Table 5.6. Fatty acids C16 N Alcohol, 18:3ω6c, C20 N Alcohol, Cis9 Epoxy10 18:0, C 22 Primary Alcohol, C 23 Primary Alcohol, 23:0 3OH and three unidentified fatty acids with 21.681, 22.675 and 23.664 carbon length were found to be present in significantly higher amounts in mycorrhizal roots. Figure 5.3 illustrates the magnitude of these differences in concentration of these fatty acids between the non-mycorrhizal control roots and the roots of plants colonised by each of the 3 *Gigaspora* species. The other fatty acids listed in Table 5.6 did not show significant differences in the amounts between mycorrhizal and non-mycorrhizal roots. Surprisingly, fatty acid 16:1ω5c was not detected in the roots of leek plants colonised by these 3 *Gigaspora* species.

5.4.2.4 FAME Analysis of Soils

Fatty acids detected in the FAME profiles of soils of all treatments before and after a 12-week growth period are shown in Table 5.7 a, b. The differences in the amounts of fatty acids between mycorrhizal and control soils at the end of the experiment were statistically significant for all fatty acids except 16:0, 22:1ω9c and 24:1ω9c. The 20-C fatty acids were present only in mycorrhizal treatments. Fatty acid 18:1ω9c was significantly higher in soils from pots containing *Gi. margarita* and *Gi. rosea* than pots containing *Gi. decipiens*. There was an increase in the amounts of 16:1ω5c, 18:1ω9c and 20:1ω9c in the soil from pots colonised by *Gi. margarita* and *Gi. rosea* during 12-week growth period (Tables 5.7 a, b). A comparison of the difference in

the amount of selected fatty acids in soil under the control (non-mycorrhizal) plants and the soil colonised by each of the 3 *Gigaspora* species is shown in Figure 5.4.

Table 5.6. Fatty acids (μg/g root) detected in FAME profiles of the roots of leek plants colonised with three species of *Gigaspora* and in control leek plants without mycorrhizal infection. The values are means of 10 replicates for all treatments.

Fatty Acids	Control	*Gi. margarita*	*Gi. rosea*	*Gi. decipiens*	LSD	Significance (<0.05)
C16 N Alcohol	0.7	52.3	44.5	21.3	9.14	<0.001
16:0	83	511	100	8.8	228	0.189
18:3ω6c	1.6	76.7	85.3	57.9	17.3	<0.001
18:2ω6c	138	725	114	144	294	0.130
18:1ω9c	**30**	**281**	**56**	**18**	**88.6**	**0.017**
C20 N Alcohol	2.1	60.5	43.2	27	9.56	<0.001
Cis9 10Epoxy 18:0	2	298	132	40	36	<0.001
20:1ω9c	**1.3**	**17.3**	**6.7**	**0.1**	**8.52**	**0.185**
i21:0	116	494	115	156	191	0.161
C22 Primary Alcohol	4	213	149	100	34.8	<0.001
C21.681	3	641	334	109	69.4	<0.001
22:1ω9c	3.88	0	0	0.18	1.07	0.002
22:1ω3c	7.7	21.5	0	4.5	13.7	0.444
22:0	11.7	25.3	0	6.6	9.82	0.088
C23 Primary Alcohol	0	43.1	20.3	14	9.79	<0.001
C22.675	2.8	60	28.6	16.3	106	<0.001
C23.664	5	671	462	231		<0.001
23:03 OH	5	216	121	201		0.014

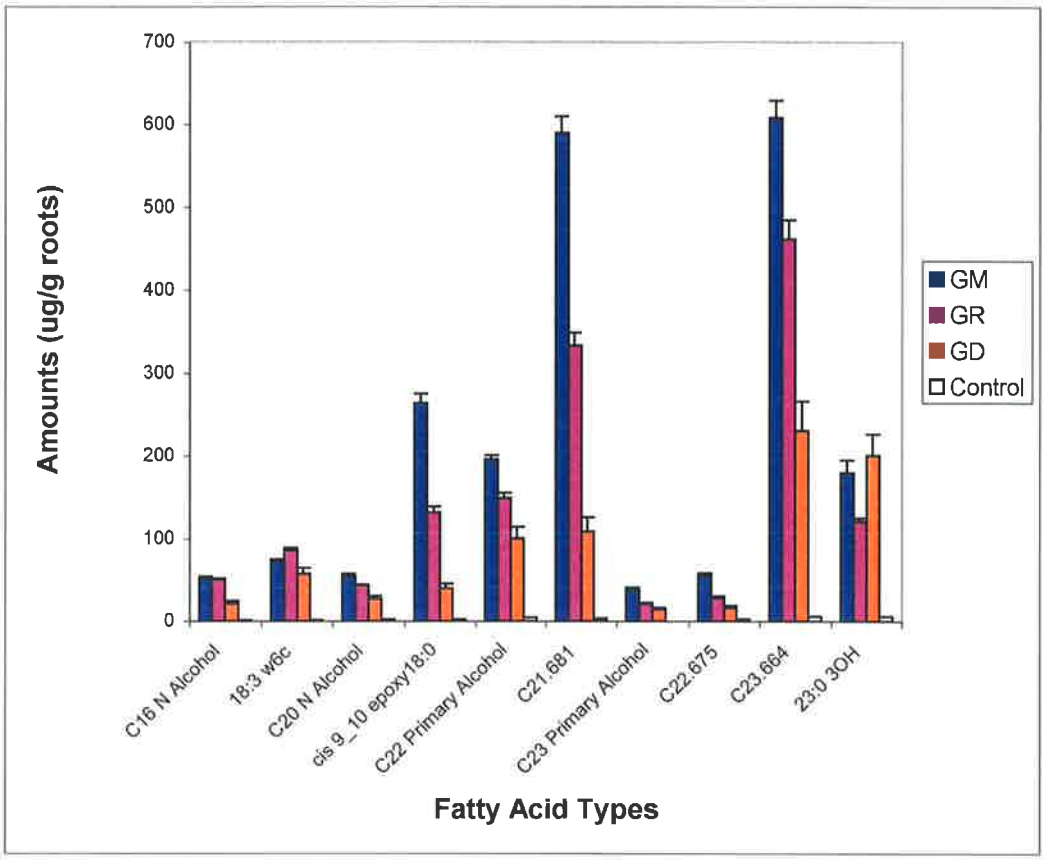


Figure 5.3 Amounts (μg) of selected fatty acids detected in FAME profiles of non-mycorrhizal leek roots and those colonised by *Gi. margarita* (GM), *Gi. rosea* (GR) and *Gi. decipiens* (GD). Each of these fatty acids was significantly higher in mycorrhizal roots than non-mycorrhizal roots (Table 5.6). Each value is a mean of 10 replicates.

Table 5.7a. Amounts (μg/g soil) of fatty acids detected in FAME profiles of the inoculum soils of four treatments (non-mycorrhizal and three AM species) at the beginning of the experiment. Each value is a mean of 10 replicates.

Fatty Acids	Control	*Gi. margarita*	*Gi. rosea*	*Gi. decipiens*	LSD	Significance (<0.05)
16:0	2.98	7.27	7.47	5.90	2.85	<0.001
16:1ω5c	**0.23**	**1.00**	**1.00**	**0.42**	**0.16**	**<0.001**
18:1ω9c	**0.59**	**3.40**	**3.41**	**1.54**	**0.32**	**<0.001**
18:2ω6c	0.12	0.82	1.09	0.96	ns	0.06
20:1ω9c	**0.00**	**0.47**	**0.47**	**0.01**	**0.06**	**<0.001**
20:3ω6c	0.04	0.20	0.25	0.11	0.03	<0.001
20:4ω6c	0	0.04	0.15	0	0.02	<0.001
20:5	0	0.02	0	0	ns	0.083
21:0	1.17	2.49	3.85	1.79	0.74	0.014
21:1ω6c	0.60	1.02	1.64	1.09	ns	0.077
21:0 2OH	0.14	0.33	0.39	0.30	0.06	0.011

Table 5.7b. Amounts (μg/g soil) of fatty acids detected in FAME profiles of the soils of four treatments (non-mycorrhizal and three AM species) after 12-weeks growth. Each value is a mean of 10 replicates.

Fatty Acids	Control	*Gi. margarita*	*Gi. rosea*	*Gi. decipiens*	LSD	Significance (<0.05)
16:0	6.24	8.89	7.43	5.10	ns	0.064
16:1ω5c	**0.16**	**2.36**	**1.23**	**0.38**	**0.42**	**<0.001**
16:1ω9c	0.63	0.71	1.63	1.63	0.42	<0.001
18:1ω9c	**5.53**	**13.09**	**13.12**	**3.51**	**4.86**	**<0.001**
18:1ω9t	0.94	1.74	1.76	0.95	0.55	0.003
18:2ω6c	3.14	3.75	7.30	2.02	2.78	0.004
18:3ω6c	0.11	0.37	0.52	0.38	0.19	<0.001
20:1ω9c	**0**	**1.32**	**0.53**	**0**	**0.42**	**<0.001**
20:2ω6c	0	0.69	0.21	0.02	0.18	<0.001
20:3ω6c	0	0.17	0.06	0.08	0.12	0.058
20:4ω6c	0	0.40	0.37	0.25	0.19	<0.001
20:5ω3c	0	0.31	0.09	0	0.08	<0.001
22:1ω9c	0	0.05	0	0.03	ns	0.577
24:1ω9c	0	0	0	0.01	ns	0.408

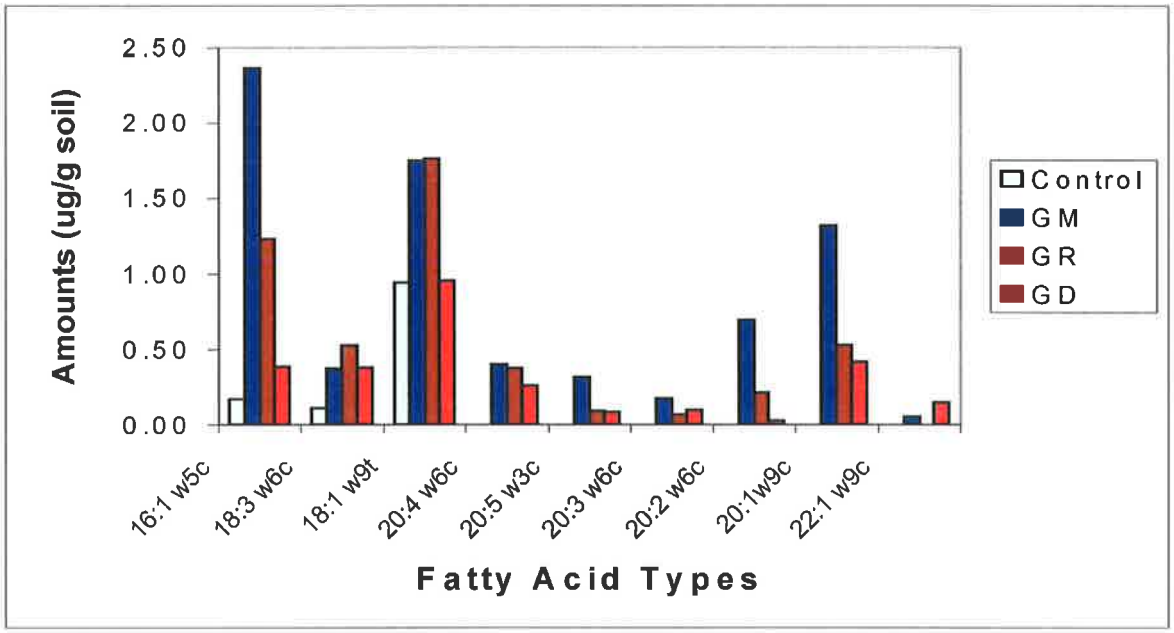


Figure 5.4 Amounts (μg/g soil) of selected fatty acids detected in FAME profiles of soils form under non-colonised control leek plants and those colonised with *Gi. margarita* (GM), *Gi. rosea* (GR) and *Gi. decipiens* (GD) after 12 weeks growth. Each value is a mean of 10 replicates.

5.5 Discussion

5.5.1 Fatty Acid Analysis of Spores

FAME analysis indicated a linear trend between the concentration of 16:1ω5c and increasing spore number in all AM species tested. This relationship between amount of 16:1ω5c and spore number was more evident with *G. mosseae* and *Gi margarita* than it was with *S. calospora* and *G. coronatum*. The lack of clear relationship with *S. calospora* and *G. coronatum* could be due to variation in spore size and spore age in the samples tested. Of the four AM fungi tested, *S. calospora* had the largest spores, whilst *G. coronatum* had the smallest spores (Figure 5.2a). The amount of 16:1ω5c present in the spores also varied and was highest in the smaller *G. coronatum* spores. Data was not collected on the range of spore sizes within a species but this is likely to vary with spore age, with spore size likely to increase with spore age. Thus in any sampling of spores a range of spore sizes and ages will be represented giving rise to variation in the amount of 16:1ω5c detected. These variations could be greater with species producing large spores or small spores containing high concentrations of 16:1ω5c. Another example of this kind of variation can be seen when one compares the recovery of 16:1ω5c from 100 spores of *G. coronatum, G. mosseae* and *S. calospora* (Tables 5.1, 5.2 and 5.3).

The capacity to recover most of the fatty acids theoretically contained in the spores of different AM species when a known number of spores was added to a soil-sand mix, provided good evidence for the quantitative recovery of fatty acids from this medium using the FAME technique. This is important as it lends support to the potential use of FAME analysis to detect and quantify the amount of biomarker fatty acids such as 16:1ω5c in the soil.

If standardised, the estimated amount of 16:1ω5c per spore could be useful in estimating the biomass of AM fungi in soil. This per-spore concentration varied between species and did not depend on the weight of individual spores in the 4 AM species studied. For example, one spore of *G. coronatum* with an average weight of 34 μg contained 0.48 μg of 16:1ω5c, while a spore of *S. calospora* with an average weight 110 μg contained 0.33 μg of 16:1ω5c (Figure 5.2). Thus when comparing

species of AM fungi, it is suggested that the presence of 16:1ω5c will not depend on the size of the spores but probably on the inherent capacity of a particular species to produce and store this fatty acid.

The total amount of extractable FAMEs was highest for the spores of *Gi. margarita* (Tables 5.1, 5.3). In these experiments, fatty acid 16:1ω5c contributed only about 5% of the total FAMEs recovered from the *Gi. margarita* spores, the rest being contributed by 18-C and 20-C fatty acids (Table 5.1). This is in contrast to the results obtained in Chapter 3 (Table 3.3) where 16:1ω5c contributed 43% of the total FAMEs extracted from this species. The reason for this variation could be explained by differences in the mode of collection of samples used in Chapter 3 and this chapter. In Chapter 3, the samples of spores comprised a certain weight of extracted spore mass, which inevitably must have had some amounts of hyphae that could not be separated from the spores during spore extraction. Spores in these samples were not hand picked as was done in all the other spore analyses, therefore, that presented the possibility of contamination of the sample not only with the hyphae but also with the spores of the other species. Furthermore, the differences in spore age (and hence the lipid content) may also have an unknown contribution of variation in spore size and bio-volume of spores in this analysis. A similar result was obtained with the spores of *S. calospora* with 16:1ω5c contributing 50% of the total FAMEs in this experiment in contrast to only 10% in the results obtained in Chapter 3, which also coincided with the poor growth of leek plants with *S. calospora* (Table 3.2). However, with the *Glomus* spp. tested there was better agreement with proportion of 16:1ω5c in the total FAMEs extracted being close to 50% in this experiment (Table 5.1) and in the earlier experiment (Table 3.3).

An important objective of this experiment was to confirm the results of a previous experiment (Chapter 3) in demonstrating which fatty acids detected in the FAME profiles of spores, could be used to differentiate between AM fungal species at the genus and family level. Fatty acid 16:1ω5c was confirmed as a general bio-marker for AM fungi, and 18:1ω9c was confirmed as a fatty acid that has potential to differentiate *Gigaspora* species from species of *Glomus* and *Scutellospora*. *Gigaspora* species contained 30-80 times more 18:1ω9c than species of *Glomus* and

Scutellospora confirming the earlier findings in Chapter 3 and previous studies of the other researchers (Graham *et al.* 1995). According to Beilby (1980) and Nordby *et al.* (1981), 18:1ω9c is a dominant fatty acid of AM fungi, but its potential value in differentiating AM species especially *Gigaspora* from other AM species, has not previously been reported.

Fatty acid 20:1ω9c and other related 20-C polyunsaturated fatty acids were of interest because they were generally detected only in the spores of *Gi. margarita* (Tables 5.1, 5.2). This was further confirmed in the data presented in Table 5.3, where the amounts of these fatty acids reflected clearly the number of *Gi. margarita* spores added to the soil-sand mix. These fatty acids were also present in the spores of *Gi. rosea* (Table 5.5). Hence these fatty acids may have diagnostic importance at the genus level, although this will have to be confirmed by studies with more *Gigaspora* species. 20-C fatty acids have previously been reported from *Gigaspora* spp (Graham *et. al.* 1995; Johansen *et. al.* 1996). These fatty acids are also reported to occur in algae and protozoa but have not been reported from non-AM fungi and are present only in low amounts in bacteria (Federle 1986; Lechevalier & Lechevalier 1988). One has to be careful in considering the benefits of using these fatty acids as markers in field soils or algae-infested pots in experimental systems, since it is easy to get algae infection by over-watering the pots or improper light conditions. Also, as small amounts of 20-C fatty acids were detected in the roots of non-mycorrhizal leek plants (Table 5.6), they may have limited value for detection of *Gigaspora* species in colonised plant roots.

5.5.2 FAME Analysis of *Gigaspora* Species

In the experiment set up to detect differences in the FAME profiles of spores, colonised soil and colonised roots of three *Gigaspora* species, only two of the species (*Gi. margarita* and *Gi. rosea*) established an effective mycorrhizal association with the host leek plants. The plants inoculated with the third species, *Gi. decipiens* were poorly colonised and showed no mycorrhizal growth responses. FAME profiles of the soil used to inoculate the plants with *Gi. decipiens* revealed very small amounts of mycorrhizal fatty acids eg. 16:1ω5c, 18:1ω9c and 20:1ω9c (Table 5.7a), indicating

that the soil inoculum contained low amounts of *Gi. decipiens* which may have resulted in the poor development of the mycorrhizal association. If one used the amount of 16:1ω5c estimated per spore for *Gi. margarita* as a possible reference for the amount of 16:1ω5c in spores of *Gi. decipiens*, it would be concluded that there were <2 spores / g soil in the *Gi. decipiens* inoculum soil compared to 5 spores / g soil in the *Gi. margarita* inoculum soil. This difference could partly explain the poor performance of the *Gi. decipiens*, ie. there were insufficient spores in the inoculum soil to establish an effective symbiosis during the course of the experiment. Other soil factors including the type of soil used in the experiment or the soil pH may also have contributed to the poor growth of *Gi. decipiens*.

This experiment was performed to verify the correlation of particular fatty acids eg. 18:1ω9c, 20:1ω9c, 20:2ω6c and 22:1ω9c with the spores of *Gi. margarita*, and to confirm whether these fatty acids were found in other members of the genus *Gigaspora*. The presence of these fatty acids in the spores of *Gi. margarita* was confirmed (Table 5.5) and they were also found in large amounts in the spores of *Gi. rosea*. Two of these fatty acids (18:1ω9c and 20:1ω9c) were also detected in plant roots and soil colonised by these two species. Hence it is not possible to use these fatty acids for taxonomic differentiation between these two *Gigaspora* spp. However, the consistent detection of these fatty acids within *Gigaspora* species confirms their usefulness in differentiating *Gigaspora* spp. from other fungi.

Whilst it was not possible to test spores of *Gi. decipiens* in this experiment, the soil and roots of plants inoculated with this AM species were analysed. The plant roots did not contain levels of 18:1ω9c and 20:1ω9c above those detected in non-inoculated control roots but the soil containing *Gi. decipiens* used to inoculate the plants did contain significant amounts of these two fatty acids (Table 5.7a). Hence it appears that *Gi. decipiens* also contains fatty acids characteristic of *Gigaspora* species. But due to poor growth of *Gi. decipiens* it can not be concluded with certainty.

Some previous studies of FAME profiles of *Gigaspora* species have been published. Graham *et al.* (1995) studied 53 isolates of 24 species of Glomalean fungi including 12 isolates of *Gigaspora* belonging to four species (*Gi. albida, Gi.*

gigantea, Gi. rosea, and *Gi. margarita*). Some of their observations agreed with the results obtained here. For example, they found 20:1ω9c to be present only in *Gigaspora* species at levels ranging from 8-15% of total fatty acids of 9-20 carbon length; it was absent in isolates of *Acaulospora, Glomus* and *Scutellospora.* Similarly, they observed high levels of 18:1ω9c (38-48%) in isolates of *Gigaspora.* Bentivenga & Morton (1994) also found fatty acid 18:1ω9c to contribute from 35-55% of the total fatty acid profile in *Gigaspora* isolates but less than 10% in other AM fungal isolates.

In contrast, Bentivenga & Morton (1994) and Graham *et al.* (1995) found that 16:1ω5c was either absent or <2% of total fatty acids in isolates of *Gigaspora.* In the present study, and including the results obtained in Chapter 3, 16:1ω5c was found to make up from 5-40% of the total FAMEs extracted from the spores of *Gi. margarita* and from 2-10% of the total FAMEs extracted from the spores of *Gi. rosea.* In addition 16:1ω5c was detected in the FAME profiles of the soil colonised by *Gi. margarita* and *Gi. rosea* (Tables 3.5, 5.7a, 5.7b) and on one occasion in plant roots colonised by these AM fungi (Table 3.4). Its detection in colonised plant roots was erratic, as it was not detected in the colonised roots in the experiment reported in this Chapter (Table 5.6). It was important to note that 16:1ω5c was either not detected or detected in very low amounts in the PLFA profiles of roots and soil colonised by *Gi. margarita* and *Gi. rosea* (Tables 3.4, 3.5). The fatty acid extraction protocol used may therefore influence whether or not this fatty acid is detected when present in roots or soils. However, since all spore analyses were carried out on hand picked spores only, any chance contamination can entirely be ruled out and the reliability of the results could be thoroughly trusted. Hence, the data suggests that whilst this fatty acid is present in the spores of *Gi. margarita* and *Gi. rosea*, it may be present in only very low concentrations in the hyphae of these two AM fungi

5.6 Conclusion

From the above study the following conclusions are drawn:

- Amounts of fatty acid 16:1ω5c were directly related to the number of spores of *G. coronatum, G. mosseae, Gi. margarita,* and *S. calospora* whether with spores only or spores in the background of soil.
- The concentration of 16:1ω5c in individual spores varied with AM species but it was not related to the weight of individual spores.
- The concentration of 16:1ω5c per spore could be used to estimate the total biomass of spores of an individual AM species in soil.
- Crushing of spores had a small but mostly non-significant effect on the yield of fatty acids. The yield was slightly decreased with crushing due to possible loss.
- Fatty acids 18:1ω9c and 20:1ω9c were confirmed as potential biomarkers to differentiate *Gigaspora* species from other AM fungi, but may not be useful for differentiating between individual species of *Gigaspora.*

Chapter 6: Can Fatty Acids be used to Measure Mycorrhizal Infectivity in Field Soils?

6.1 Introduction

In the previous experiments there was a clear indication that it would be possible to utilise the FAME technique for the detection of AM fungi in soils and plant roots, as well as for the identification of some selected AM fungal species eg. species of *Gigaspora.* There was also an indication that the amounts of propagules of AM fungi in the soil could be quantified on the basis of information concerning the amount of selected fatty acids (in particular 16:1ω5c) present in the spores of different AM fungal species. All these results lead to the possibility of developing the FAME technique into a tool for monitoring the activity of AM fungi in soil and plant roots. However, as all the previous experiments were conducted in controlled environment conditions, it was necessary to test the methodology in the field conditions. Field soils provide the additional challenge of whether or not it is possible to discern a selected biomarker fatty acid against a background of fatty acids derived from the different microorganisms and organic matter present in the soil.

Based on current literature evidence (discussed in Chapter 3), the fatty acid with the most potential of being used as a biomarker for AM fungi (ie. 16:1ω5c) is not commonly found in plant tissues and occurs rarely in other soil microorganisms. The results of the FAME analysis of 10 different uncolonised plant species, known to normally form mycorrhizal associations (Chapter 3), also confirmed that this fatty acid is not a common constituent of plant tissues nor present in rhizosphere organisms attached to the roots. This evidence suggests that detection of 16:1ω5c in soils would generally be a good indication of presence of AM fungi in that soil.

In this Chapter, FAME analysis is used to detect 16:1ω5c in three different field soils. This measure of AM infectivity of the soils is then compared with the determination of AM infectivity of the same soils using conventional microscopic techniques, based on determination of spore number and % root colonisation of

different host plants. In addition the effect of serial dilution of the field soils on the amounts of fatty acids detected (particularly 16:1ω5c) was tested to further validate the use of the FAME technique and also to evaluate the limits of detection of this fatty acid in soil.

Part of the work described in this Chapter was done in collaboration with Dr Evelina Facelli who collected the soil samples and determined the AM infectivity of the soils using % colonisation measurements and spore counting. The author duly acknowledges this contribution.

6.2 Aims

The main aims of this experiment were:

1. To obtain FAME profiles of three different field soils having different levels of AM infectivity.
2. To obtain FAME profiles from four levels of dilutions of field soils to confirm the applicability and efficacy of the fatty acid technique in field soils and to check the limit of detection of 16:1ω5c in diluted samples of soil.
3. To estimate the AM infectivity of field soil by counting the number of AM fungal spores and % root colonisation of 4 different plant species and compare these estimates with an estimate of AM infectivity based on soil FAME analysis.

The following experiments were performed to achieve the above aims.

1. FAME profiles of 3 field soils
2. FAME profiles of 4 dilution levels of field soils
3. Infectivity observations of field soils by microscopic methods eg. % colonisation of trap host plants, and spore number estimations.
4. Correlation of % colonisation and spore number with the amount of fatty acid 16:1ω5c detected in the soil.

6.3 Materials & Methods

6.3.1 Field Sites and Collection of Soil Samples

Soil samples were collected from 3 sites at Wallowa Station (30 km from Murray Bridge, South Australia). Part of this property is under the Natural Heritage Scheme and thus contained areas of undisturbed abandoned pasture, pre-dominantly inhabited by native vegetation eg. species of *Eucalyptus, Melaleuca, Leptospermum, Baekea* and *Acacia*. This open woodland area with undisturbed vegetation was referred to as the 'Mallee' site. An adjacent area of land, which was cleared in the1950's and is now re-growing naturally and is dominated by natural shrub-land, was termed the 'Re-growth' site in the present experiment. The third experimental site was an area of grassland adjacent to the Mallee site, which is dominated by exotic plant species including *Marrubium vulgare, Euphorbia teraci* and *Oenothera stricta*. This was referred to as the 'Pasture' site. These adjacent areas of land, each with a different history of disturbance, provided an appropriate field-study area with naturally existing diversity in AM infectivity. Other scientists at Adelaide University, Adelaide, were also studying these sites for determination of floristic changes, successional processes and rates of regeneration of natural vegetation.

Soil was collected from 10 different locations (separated from each other by 10 meters), at each field site. At each location four samples of 500 g soil were collected. The soil samples from the 10 locations within each field site were thoroughly mixed to provide the soil used to grow four different host plants and for FAME analysis.

6.3.2 Host Plants and Maintenance of Pot Cultures

Four host plants namely, *Acacia calamifolia, Danthonia caespitosa, Trifolium subterraneum* and *Eucalyptus incrassata* (representing herbaceous, fast growing and slow growing shrubs and canopy trees) were grown in each of the three types of soils in the glasshouse. Five replicated pots containing 1.4 kg field soil each were used to grow each plant species. The pots were placed in a glasshouse (temperature variation 5-35°C) and watered three times a week. The plants were grown for 24 weeks and

then harvested. The roots of the plants were washed thoroughly before being removed for analysis.

6.3.3 Microscopic Observations

At plant harvest, spores were collected from a 100 g soil sample taken from each pot using the wet sieving method and counted on a nematode dish (see Chapter 2, section 2.8).

Root samples (1 g) were stained and examined for % root colonisation by mycorrhizal fungi using the method as described in Chapter 2 (see section 2.7).

6.3.4 FAME Analysis of Soils

Five 6 g samples of each of the three field soils were processed for FAME analysis as described in detail in section 2.9 of Chapter 2. In order to check the effect of soil dilution on the amounts of fatty acid recovered, the soil was diluted to four levels by adding sterilised sand (autoclaved at 121°C for 1 h twice, then dried at 105°C in oven). The soil dilutions were: 75 g soil + 25 g sand, 50 g soil + 50 g sand, 25 g soil + 75 g sand and 10 g soil + 90 g sand. The soil and sand were mixed thoroughly before a 6 g sample was taken from each soil dilution for FAME analysis. Five replicates of each dilution level of each soil type were processed for FAME analysis. The FAME data was subjected to a general analysis of variance.

6.4 Results

6.4.1 FAME Analysis of Soils

The amounts of fatty acids detected in the FAME profiles of each of the three field soils are shown in Table 6.1. The results showed that the concentrations of fatty acids with lower carbon number (from 9-C to 14-C) were more abundant in the Mallee soil than the other two soil types. The fatty acids with higher carbon number (15-C to 16-C), however, were more abundant in the pasture soil than the other two types. The unsaturated fatty acids of 18-C were also higher in pasture soil while the straight-chained 18-C fatty acids and all other fatty acids observed in this analysis (19-C and

20-C) were higher in the Mallee soil. There were more fungal-related fatty acids (eg. 18:2ω6c) and more fatty acids associated with AM fungi (eg. 16:1ω5c, 18:1ω9c) in the pasture soils than in the Mallee or Re-growth soils. The Re-growth soil type generally had the lowest amounts of fatty acids. The differences in amounts between all fatty acids were statistically significant at $P < 0.05$.

6.4.2 FAME Analysis of Diluted Soils

The amounts of fatty acids obtained from the four dilution levels of each soil type are shown in Table 6.2. The dilution of the soils generally caused a systematic reduction in the amounts of fatty acids detected, all differences in the values of fatty acids being statistically significant ($P < 0.001$). Mycorrhiza-related fatty acid 16:1ω5c could be detected in the lowest level of dilution of soil in Mallee and Pasture types while in Re-growth type it could not be detected below 50% dilution of the soil due to initial lower concentration in this soil type.

Table 6.1. Amounts (μg) of fatty acids detected in FAME profiles of three different field soils. Each value is a mean of five replicates.

Fatty Acid	**Amounts (μg/g)**			**LSD**	**Significance (P<0.05)**
	Mallee	Pasture	Re-growth		
C9 Dicarboxylic Acid	14.63	3.44	3.40	3.68	<.001
C10 Dicarboxylic Acid	2.62	0.57	0.51	0.44	<.001
12:0	3.57	1.87	1.29	0.80	<.001
13:0	4.08	0.30	0.44	0.73	<.001
14:0	11.14	4.51	2.62	1.65	<.001
15:0	3.55	4.85	1.30	0.66	<.001
16:0	15.43	16.46	8.33	2.43	<.001
16:1ω5c	4.42	9.57	1.09	1.77	<.001
16:1ω7c	3.68	5.99	1.89	0.65	<.001
18:0 2OH	5.51	2.07	2.19	1.45	<.001
18:1ω9c	6.45	15.54	6.14	4.1	<.001
18:1ω9t	1.81	3.15	0.94	0.85	<.001
18:2ω6c	3.6	14.9	2.9	7.77	0.005
19:1ω8t	6.19	3.22	3.08	1.56	<.001
20:4ω6c	0.07	1.34	0	0.20	<.001
20:5ω3c	0	0.98	0	0.09	<.001
i21:0	13.1	5.81	9.96	1.97	0.002
21:0	15.64	6.29	8.30	5.14	0.001
21:1ω9c	8.48	2.35	2.57	3.28	<.001
22:1ω9c	0.77	0.06	0.43	0.56	0.039
C23.283	2.26	1.12	1.03	0.42	<.001

Table 6.2. Amounts of fatty acids (μg) detected in FAME profiles of four dilutions of three different field soils. Values are a mean of five replicates of each treatment. The numbers 10, 25, 50 and 75 represent the weight (g) of soil in a 100 g soil/sand mix.

Fatty Acids	Mallee				Pasture				Re-growth				Significance
	10	25	50	75	10	25	50	75	10	25	50	75	P<0.05
12:0	0.45	1.89	2.93	2.82	0	0.5	1.02	1.5	0.45	0.84	1.44	1.69	<0.001
13:0	0	0.21	0.69	1.59	0	0	0	0	0	0	0.27	0.55	<0.001
14:0	1.70	5.34	7.37	8.07	0.62	1.23	2.47	3.35	0.57	1.19	2.15	3.21	<0.001
15:0	0	0.66	1.75	2.55	0.10	1.34	2.69	4.20	0	0.08	0.77	1.52	<0.001
16:0	3.76	10.66	14.05	14.2	2.86	6.64	12.5	14.4	1.74	3.41	6.46	9.65	<0.001
16:1ω5c	0.28	2.58	3.02	4.0	1.04	2.63	5.21	6.79	0	0	0.67	1.11	<0.001
16:1ω7c	0.21	2.40	3.39	3.51	0.80	1.98	3.28	4.84	0	0.14	1.68	1.92	<0.001
18:0 2OH	0.27	2.79	3.99	5.14	0	0	1.03	1.42	0	0.10	1.60	1.78	<0.001
18:1ω9c	1.56	3.51	5.41	7.49	0.93	3.13	6.75	8.27	1.08	2.49	5.45	7.75	<0.001
18:1ω9t	0	0.59	1.74	1.83	0.25	1.32	2.30	2.95	0	0	0.51	1.22	<0.001
18:2ω6c	0.08	1.08	3.0	3.81	0.50	2.86	4.93	4.33	0.19	0.88	2.76	3.32	<0.001
19:1ω8t	0.40	3.45	4.08	5.52	0	0.75	1.39	2.41	0	0.72	2.44	2.73	<0.001
i21:0	1.92	6.06	10.67	13.8	0.25	1.24	2.61	3.43	1.06	1.95	7.06	7.22	<0.001
21:0	1.99	6.25	10.82	12.8	0.15	1.25	2.46	3.61	0.91	1.59	5.53	6.14	<0.001
21:1ω9c	0.28	3.30	3.53	5.74	0	0	1.02	1.43	0	0.95	2.91	2.47	<0.001
C21.252	0	2.26	3.65	4.80	0	0.56	1.78	3.15	0	0.90	2.95	3.43	<0.001
C24.971	2.35	4.66	5.37	6.67	0.70	1.40	1.52	1.89	1.14	2.17	3.54	3.83	<0.001
C25.316	0.38	0.61	1.12	1.70	0.17	0.84	1.13	1.55	0.06	0.51	1.16	2.05	<0.001

6.4.3 AM Infectivity Determined by Microscopic Methods

6.4.3.1 Spore Number

The total number of spores (including young and unidentified spores) obtained from each sample of 100 g soil of each plant-soil combination after 24 weeks growth is shown in Figure 6.1. The total number of spores counted was highest in the pasture soil compared to the other two soil types and highest under clover compared to the other 3 host plants. The Mallee soil contained the least number of spores with all host plants, while the Re-growth soil had only a slightly higher number. There was greater variability in spore number associated with soil type than with plant host type.

6.4.3.2 Percent Colonisation of Roots

The average percent of root length colonised by mycorrhizal fungi for each of the four host plants in the three field soils is shown in Figure 6.2. Similar to the spore number observations, the values for percent colonisation of roots were higher for the host plants growing in the pasture soil than in the other two soil types. Clover was also the most extensively colonised of the 4 different host plants. The percent colonisation varied from a minimum of 25% (Acacia in Re-growth soil) to a maximum of 71% (Clover in Pasture soil). There was less variability in % root colonisation than there was in spore number. The Eucalyptus (normally ectomycorrhizal) plant roots were observed with AM fungal hyphae in roots and the % colonisation data presented here is with AM fungi.

6.4.4 Relationship between AM Infectivity Determined by Microscopic Observations and Soil FAME Analysis

The correlation of the average amounts of fatty acid 16:1ω5c in soils with (a) % colonisation in roots of four host plants and (b) with spore numbers in the soils with four host plants is shown in Figure 6.3. In all host types, a strong correlation existed between the amount of 16:1ω5c in soil, % colonisation in roots and the spore number in soils, with R^2 values ranging from 0.57-0.98 (Figure 6.3).

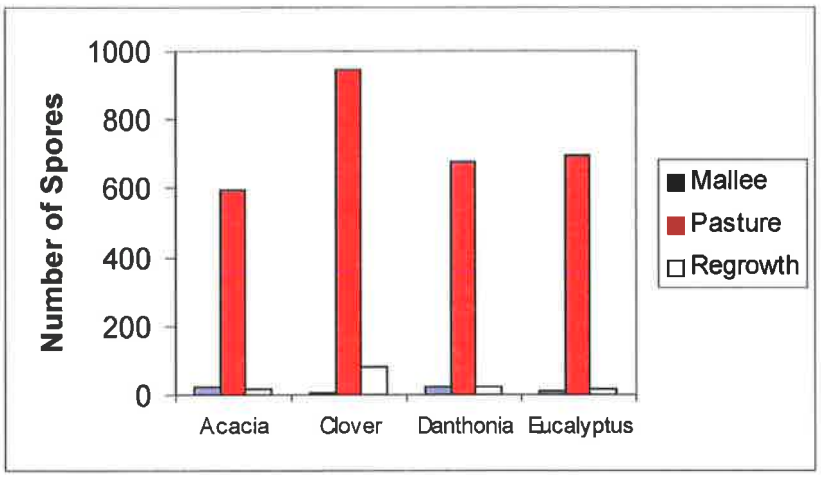


Figure 6.1. The number of spores collected from 100 g soil under four host plants in three soil types after 24 weeks of growth. Each value is a single replicate. (Data collected by Dr Evelina Facelli).

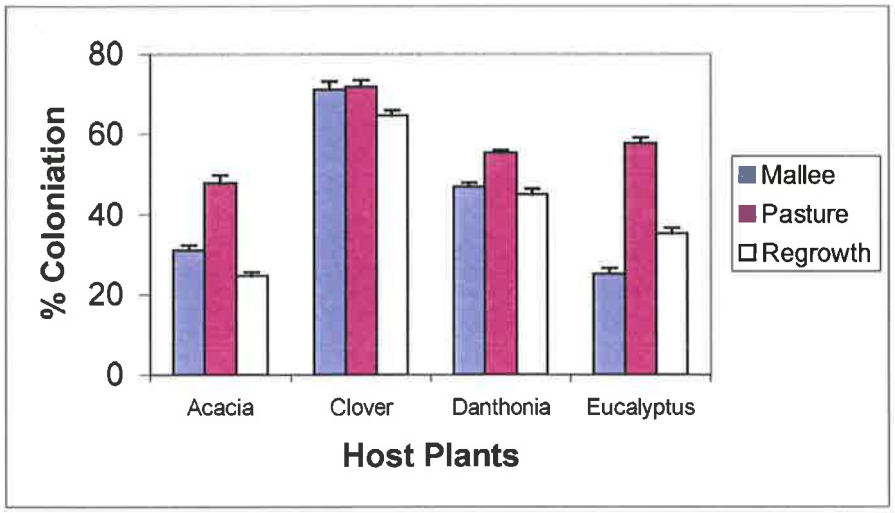
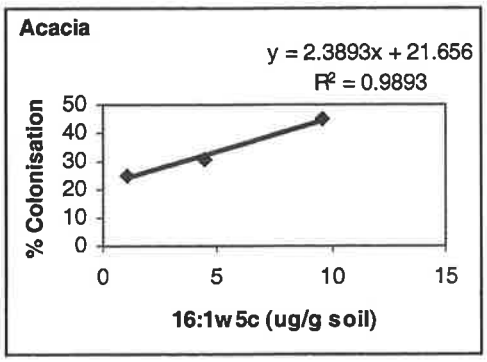
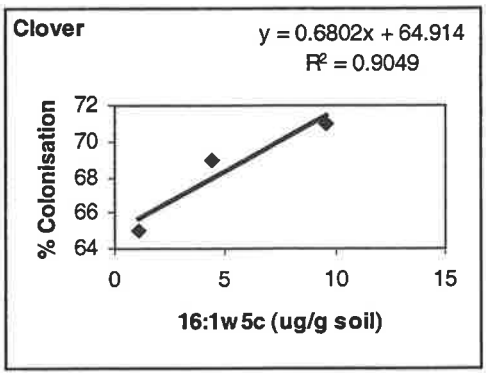


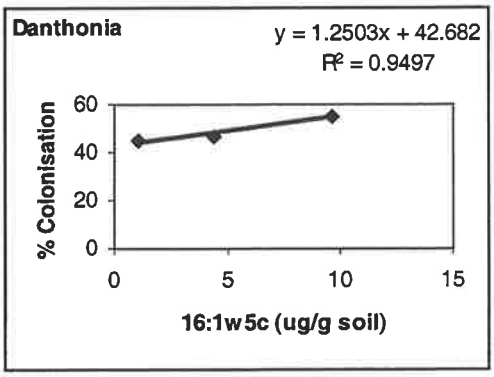
Figure 6.2 Percent colonisation by AM fungi of the roots of four host plants in three field soils. Values are an average of 10 replicates of each treatment. (Data collected by Dr Evelina Facelli).

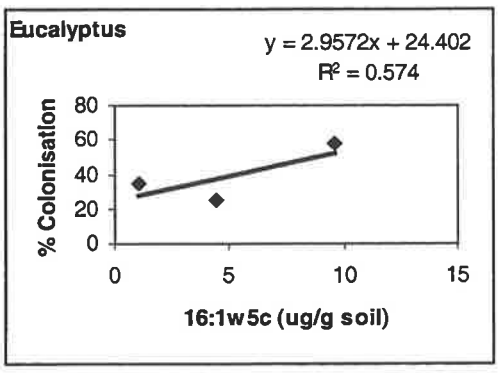
Figure 6.3 Correlation between amount of 16:1ω5c (μg/g soil) with (a) % mycorrhizal colonisation of the roots of Acacia, Clover, Danthonia and Eucalyptus, and (b) number of spores (No. / 100g soil) in soil under these plants.

(a)

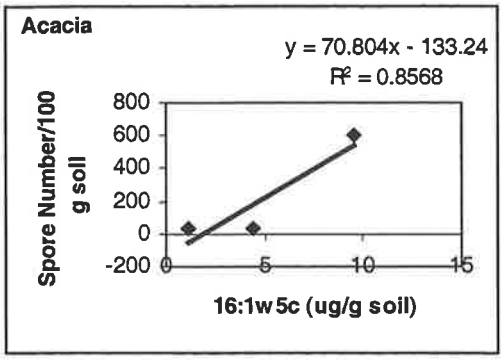

Acacia
y = 2.3893x + 21.656
R² = 0.9893
% Colonisation
50
40
30
20
10
0
0
5
10
15
16:1w5c (ug/g soil)

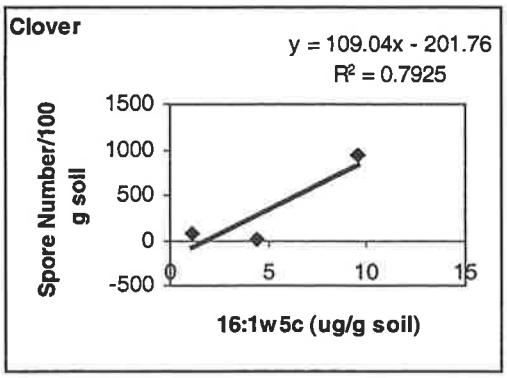

Clover
y = 0.6802x + 64.914
R² = 0.9049
% Colonisation
72
70
68
66
64
0
5
10
15
16:1w5c (ug/g soil)

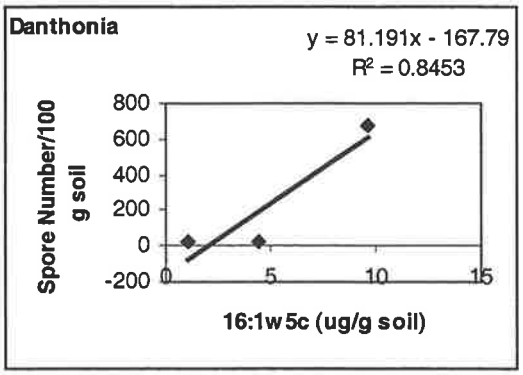

Danthonia
y = 1.2503x + 42.682
R² = 0.9497
% Colonisation
60
40
20
0
0
5
10
15
16:1w5c (ug/g soil)


Eucalyptus
y = 2.9572x + 24.402
R² = 0.574
% Colonisation
80
60
40
20
0
0
5
10
15
16:1w5c (ug/g soil)

(b)


Acacia
y = 70.804x - 133.24
R² = 0.8568
Spore Number/100 g soil
800
600
400
200
0
-200
0
5
10
15
16:1w5c (ug/g soil)


Clover
y = 109.04x - 201.76
R² = 0.7925
Spore Number/100 g soil
1500
1000
500
0
-500
0
5
10
15
16:1w5c (ug/g soil)


Danthonia
y = 81.191x - 167.79
R² = 0.8453
Spore Number/100 g soil
800
600
400
200
0
-200
0
5
10
15
16:1w5c (ug/g soil)

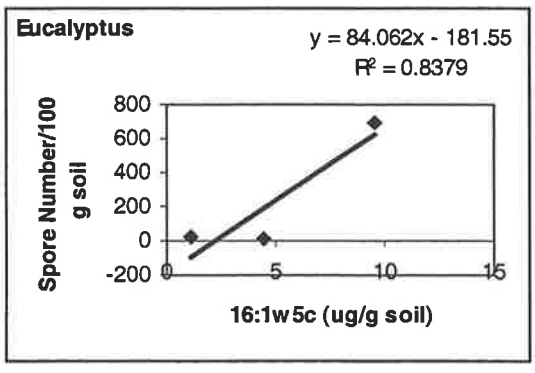

Eucalyptus
y = 84.062x - 181.55
R² = 0.8379
Spore Number/100 g soil
800
600
400
200
0
-200
0
5
10
15
16:1w5c (ug/g soil)

Table 6.3 A list of common fatty acids and their amounts (%) associated with a variety of groups of organisms as documented by various authors.

Fatty Acids	Amounts (%) From	Groups of Organisms	Authors
12:0	3 (totFA)	Gram –ve Bacteria	Thompson *et al.* 1993
14:0	5		Jarvis *et al.* 1996
16:0	27		
cy17:0	9		
cy19:0	25		
16:1ω7c	28		
18:1ω7	38		
i15:0,	31 (totFA)	Gram +ve Bacteria	Raffel *et al.* 1996
a15:0	4		
i14:0	6		Frostegard & Bååth
16:0	5		1996
i17:0	9		
a17:0	1		
cy19:0	9		
15:0	2 (PLFA)	Actinomycetes	Tunlid *et al.* 1989
16:0	6		
i16:0	52		
17:1ω8c	19		
16:1ω7c	4		
16:0	12 (totFA)	Basidiomycetes	Müller *et al.* 1994
18:2ω6c	79		Stahl & Klug 1996
18:1ω9c	2		Radwan *et al.* 1996
16:0	34 (PLFA)	Zygomycetes	Cooper & Lösel 1978
18:0	2		Pacovsky & Fuller
16:1ω7c	6		1988
16:1ω5c	16		Jabaji-Hare 1988
18:1ω9c	5		Bentivenga & Morton
18:1ω7c	27		1994
18:2ω6c	2		Olsson *et al.* 1997
20:5	6		Gasper *et al.* 1997
			Larsen *et al.* 1998
			Jansa *et al.* 1999
16:0	27 (PLFA)	Deuteromycetes	Larsen *et al.* 1998
18:0	4		
16:1ω7c	3		
18:1ω9c	14		
18:2ω6c	48		
16:0	34 (PLFA)	Eukaryotic plant roots	Wellburn *et al.*1994
18:1ω9c	4		
18:2ω6c	17		

6.5 Discussion

This experiment was performed to determine if fatty acid analysis could be used to estimate the infectivity of AM fungi in field soils and if the lengthy microscopic methods currently used for this purpose could be replaced or supplemented by soil FAME analysis. Soil infectivity levels observed by microscopic methods (spore number and % mycorrhizal colonisation of plant roots) were strongly correlated with the amount of fatty acid 16:1ω5c detected in the soil FAME profiles. The spore number counts and the % colonisation measurements showed that the pasture soil had highest amounts of AM propagules irrespective of the host plant used for the assay. This difference was correlated with the FAME analysis, where the pasture soil was found to have highest amounts of fatty acids associated with the AM fungal species (16:1ω5c, 18:1ω9c). Both methods clearly showed that the Mallee and Re-growth soils had lower AM infectivity than the pasture soil.

Some limited comment can be made about the overall composition of the FAME profiles of each of the field soils. The large amount of fatty acids known to be associated with fungi (eg. 18:2ω6c) and also of 16:1ω5c (Table 6.3), are probably associated with the diverse root biomass in this soil providing large inputs of organic matter and nutrients to support a large and active microbial biomass. In contrast, the comparatively lower amounts of these fatty acids in the Mallee and Re-growth soils reflect a lower and possibly less fungal dominated microbial biomass which may be associated with the comparatively slow regeneration of vegetation at these two sites. In last two decades, some growth of the shrub land in the protected Mallee area has been observed but it is deemed slow. Although the amounts of lower carbon fatty acids (C-9-C14) were higher in Mallee soil than the other two types, a total lack of C-17 and lower amounts of C-15 fatty acids in Mallee soil indicated a lack of occurrence of beneficial bacteria. Generally, C-14 fatty acids are considered the major fatty acids in the nodules with nitrogen fixing bacteria (Maudinas *et al.* 1982) while C-15 and C-17 are indicative of Gram +ve and Gram –ve bacteria (Frostegard & Bååth 1996) (Table 6.3). A list of previously published reports of association of different fatty

acids with different organisms is compiled here (Table 6.3), which is vital for any field study of this nature.

The soil dilution experiment brought about the expected systematic reduction in the amounts of all fatty acids in the soil FAME analysis, providing further validity for the use of this technique for analysis of field soils. It has also confirmed the applicability of this technique even in soils, which may not have very high concentrations of the fatty acids. More importantly, this experiment has confirmed the successful application of FAME technique for soils in the field. Most previous research which has utilised fatty acid analysis for the detection of AM fungi in soils, has been based on detection of 16:1ω5c using PLFA analysis (Olsson *et al.* 1999). In this Chapter as in Chapter 3 of this thesis, it has been shown that FAME analysis is as good if not better than PLFA analysis for the detection of this fatty acid in soils. The observations of this experiment have also indicated a possibility of replacing or supplementing time consuming and laborious microscopic techniques with quicker and time efficient FAME analysis for measuring the infectivity levels of soils in the field.

6.6 Conclusions

From this study the following conclusions were drawn:

- There was a strong positive correlation between the AM infectivity of 3 field soils determined by conventional microscopic techniques (spore number, % root colonisation) and the amount of 16:1ω5c detected in FAME profiles of the soils. There was similarity in the results of infectivity estimations of field soil by microscopic methods and FAME analysis technique.
- On this basis it is suggested that detection of 16:1ω5c in soils using FAME analysis could be used to provide a semi-quantitative estimate of AM infectivity in soils.
- Three different soil types could also be differentiated on the basis of their fatty acid profiles by FAME analysis.

Chapter 7: General Discussion and Summary of Results

7.1 Introduction

The general aim of this project was (a) to determine if fatty acids could be used to detect, identify and differentiate AM species in the roots of host plants or in soils, and (b) to investigate if fatty acid techniques could be used to measure the biomass, whether of infective propagules or total biomass of all propagules of AM fungi in roots or soil. The existing traditional methods of detection, identification and biomass estimation of AM fungi were also studied so that these could be compared with the new techniques based on fatty acid analysis of AM fungi. The multifarious influence of AM fungi on plant growth is well known and documented (Smith and Read 1997). Furthermore, AM species and strains differ in their ability to enhance plant growth and some plant species appear to be totally dependent on the presence of AM fungal propagules in soil for establishment and growth (Smith *et al.* 1998). This makes them key microorganisms to be monitored in both natural and agricultural ecosystems. However, currently available methods of detection and identification of fungi are severely limited. According to an estimation of Hawksworth (1991) greater than a million fungal species remain undetected with the available techniques, majority of which are soil fungi. The problems experienced with the traditional methods of monitoring AM fungi in soils or plant roots (outlined in Chapter 1), indicate that effort is warranted to discover improved biochemical approaches. The abundance of lipids in AM fungal cells and the proposed uses of fatty acid analysis as a tool in studying AM fungi justified investigating this approach in this project.

Generally two types of lipids are associated with AM fungi; phospholipids and neutral lipids (Chapter 1). This raised the question of whether studying total cellular lipids (FAME analysis) or specific lipid types (PLFA / NLFA analysis) would provide better information in relation to the aims? Both methods were tried and compared in the beginning of this project (Chapter 3). This resulted in the selection of the method based on FAME that gave the most comprehensive and reliable information, and this method was then used in all the subsequent studies.

It was also important to investigate if there was agreement between the currently used traditional microscopic techniques and the newer fatty acid techniques. If there was agreement, the traditional techniques could be replaced

or supplemented with the more rapid fatty acid techniques. The two types of techniques were compared in Chapters 3, 4 and 6 and were found to be in general agreement.

In order to determine if fatty acid analysis for the detection and quantification of AM fungi was applicable in a field situation, as well as in controlled pot experiments, the FAME technique was tested in field soils (Chapter 6).

In this final chapter the findings of all the experiments are integrated and discussed in the light of the aims of this project. The discussion is centred on the following main areas:

- Can fatty acid techniques be reliably used for studying AM fungi? Which of the two techniques used in this project - FAME and PLFA / NLFA is more efficient?
- Can we use these techniques for detection, identification and differentiation of species of AM fungi at the family and species levels?
- Can we use these techniques for measurement of the biomass of AM fungal propagules?
- How do these techniques compare with the traditional techniques of detecting and quantifying AM fungi? Would we be able to replace the traditional methods with the new techniques or supplement them by using both concurrently?

7.2 Reliability of Fatty Acid Techniques and FAME versus PLFA / NLFA

The first stage of using a technique in any study should be to ensure the reliability of the technique. While literature about previous similar studies help in providing important information, it is always best to test some of the more important aspects of the technique, if possible in the time frame of the project. In this case it was known from the literature that both FAME (for total lipids) and PLFA / NLFA (for phospholipids or neutral lipids) are successfully used for identifying microorganisms, including bacteria, fungi and AM fungi (Frostegard *et al.* 1993; Graham *et al.* 1995; Olsson *et al.* 1995; Stahl and Klug 1996). Fatty acid profiles of AM fungi had also been found to be stable and heritable through many generations (Bentivenga & Morton 1994) in pot cultures or storage and had also been shown to be unaffected by host plant type.

But there were some aspects of the techniques that were tested during this project and have not been reported before. For example, in Chapter 3, the roots of 10 commonly used host plants were tested and the absence of fatty acid 16:1ω5c and some 20-C fatty acids in all of them confirmed the assumption that these fatty acid were AM related and did not occur in non-mycorrhizal plant roots or closely associated organisms. In Chapter 5, the reliability of the yield of the fatty acids was checked by analysing the profiles of increasing number of spores. The yields were found to be linearly correlated with the increasing number of spores (Table 5.1). In Chapter 6, an analysis of samples from successionally diluted soil from the field, tested the reliability of the FAME technique with the field soil and the limit of detections. A systematic reduction in the amounts of all fatty acids was observed (Table 6.2), and the amounts of fatty acids could be detected up to one fourth dilution level of the soil in most cases.

This was the first study of its kind, which looked at the comparative performance of the two techniques, FAME and PLFA/NLFA for the same AM species. The two techniques have been used extensively but not in conjunction with each other. In this study, FAME analysis was found to be more efficient, less time consuming and it did not involve the use of expensive or dangerous chemicals. Also, FAME profiles gave satisfactory differentiation between the roots of mycorrhizal and non-mycorrhizal treatments, whereas PLFA and NLFA profiles gave better differentiation between roots colonised by the different AM species (Chapter 3). The fatty acids detected in PLFA and NLFA profiles were highly variable and they did not allow clear interpretations in the time course experiment (Chapter 4). The reasons for this discrepancy could be related to the actual amounts of fatty acids present in these two types of lipids and / or in the case of the PLFA analysis, to the possible degradation of the phospholipids during the storage of the root and soil samples at –4°C. Phospholipids are known to degrade quickly after the death of the cells (White *et al.* 1979).

The FAME technique was found to be generally reliable and sensitive enough to detect variation in the amounts of fatty acids present in the spores of AM fungi in different experiments. For example the amount of 16:1ω5c detected per spore varied from 0.4-1.2 µg for the spores of *S. calospora*. Similar variations were also observed for other species. This could be due to the fact that the spores

were from different pot experiments where differences in spore age and size plus differences in culture conditions (eg. temperature fluctuations) might be responsible for the differences in the amounts of fatty acids stored per spore.

7.3 Use of Fatty Acid Techniques in Detection, Identification and Differentiation of AM species.

As discussed earlier, inadequacy of traditional methods of detection and identification of AM fungi has led to development of molecular methods, which are routinely used in identification, classification and characterisation of species. Molecular studies with AM fungi heavily depend on the use of PCR and are based mainly on ribosomal gene cluster. The DNA sequences within the rRNA subunit contain some extremely conserved sequences, which are used to develop PCR primers (White *et al.* 1990). Use of these primers allows the amplification of specific fungal DNA in a fixed DNA sample, especially useful in fungal-host root DNA in mycorrhizal association. But these specialised methods have several limitations, especially for mixed soil environment. A variety of naturally occurring compounds in soil eg. humic acid, tannins and lignin can interfere with PCR reaction and inhibit amplification. The Glomales-specific-primers designed so far have been discovered to be not so specific to species (Clapp *et al.* 1999; Schüssler *et al.* 2001). Also, more genetic variation was reported within the ribosomal ITS sequences from spores of the same species than spores from different species taken from different soils (Pringle *et al.* 2000), challenging the use of molecular methods in identifying AM fungi. Another drawback of using only molecular techniques is that they do not discriminate between living and dead material or active and dormant phase of fungus (Gardes & Bruns 1996). Moreover, relatively small amounts of reference sequence data are available for comparison in order to establish identity of a taxon. Hence, although it will be possible to amplify sequences from soil, the possibility of its definitive identification seems unlikely.

Fatty acid analysis has been successfully applied to the development of an identification system for bacteria (Microbial I.D. Inc.), and may have similar potential for AM fungi. This assumption is based on the fact that the spores of AM fungi contain large amounts of lipids and evidence for species-based differences in the amounts and types of fatty acids in these spores. In addition,

fatty acid analysis based on spores may offer potential in taxonomic studies of AM fungi where traditional or molecular methods are often unsuccessful.

Fatty acid 16:1ω5c was confirmed as a general biomarker for species of AM fungi (Chapters 3-6). It could be used reliably to detect the presence of AM fungi in plant roots and in soil. In addition, a number of other fatty acids were shown to be specifically linked to some genera of AM fungi suggesting that they had potential to be used for the detection and identification of specific AM fungi. For example, 20:1ω9c was found to be specifically associated with the spores of *Gigaspora* species (Chapters 3 and 5). Similarly, fatty acid 18:1ω9c was shown to be present in much larger amounts in the spores of *Gi. margarita*, compared to other AM fungi, suggesting that it may have potential for detection of *Gi. margarita* spores in the background of other AM spores. Unfortunately, this fatty acid is found in large quantities (20-40% of total lipids) in a range of other fungi (Stahl & Klug 1996), limiting its usefulness to contamination-free pot experiments. However, a combination of 16:1ω5c, 18:1ω9c and 20:1ω9c may form a useful detection tool for species of *Gi. margarita* in soil. A similar approach of using the combination of 16:1ω5c and 20:5 has been suggested by Olsson *et al.* (1995) for the detection of *Glomus* and *Acaulospora* species in soil. There were other fatty acids, which were detected in the AM species of one family. For example, fatty acid 22:1ω9c was found only in the spores of the species of the family Gigasporaceae (*Gigaspora* and *Scutellospora*) (Chapters 3 and 5), suggesting its value in differentiation of species at the family level. Fatty acid profiles have previously been found in agreement with the morphological characters used to separate the two main families of Glomales (Bentivenga & Morton 1994a; Morton & Bentivenga 1994).

However, in answer to the main question ie. whether or not the fatty acids can be used to differentiate between the species of one genus, the results of the experiment with species of *Gigaspora* (Chapter 5) suggest that this is not possible, although more species of this genus need to be tested to confirm this. Graham *et al.* (1995) reached at similar conclusions after studying 53 isolates of 24 species of Glomalean fungi, which was later confirmed by Bentivenga & Morton (1996).

7.4 Use of Fatty Acid Analysis for Estimation of the Biomass of AM Propagules

One of the aims of this study was to test if fatty acid techniques could be used to measure the biomass of AM fungal propagules, which has been found to be difficult and sometimes inaccurate with existing methods. In this project the biomass estimations of AM fungi were made in two studies of soil and in plant roots colonised by different AM species (Chapters 3 and 5). In both studies, the amounts of fatty acids16:1ω5c detected were correlated with the amount of AM fungal biomass determined by traditional methods (number of spores, % colonisation of roots by AM fungi). In all instances a positive correlation was found between the amount of 16:1ω5c detected and the biomass estimations based on spore counts and root colonisation.

The amount of fatty acid 16:1ω5c present in individual spores of 5 different AM fungi examined was also determined. This showed that the spores of *Glomus coronatum* to be very rich in this fatty acid compared to the other species (Figure 3.4). These results were confirmed in a second estimation (Chapter 5) using four AM species. In the latter experiment it was also shown that the amount of 16:1ω5c per spore did not depend upon the weight of spores. The spores of the different AM species examined differed in the amount of 16:1ω5c they contained (ranging from 0.22 μg per spore for *Gi. margarita* to 0.48 μg per spore in *G. coronatum*, Figure 5.2b). This variation probably depends upon an innate capacity of the spores of a particular AM species to store lipids. Within each species the amount of 16:1ω5c detected per spore varied in different experiments. These amounts ranged from 0.48-0.88 μg per spore in *G. coronatum,* from 1.6-3.3 μg per spore in *S. calospora* and from 0.19-0.22 μg per spore in *Gi. margarita* (Figures 5.2b and 3.4). This suggests that variations in the size or weight of spores, within a species, results in variation in the amount of 16:1ω5c detected per spore. Since this study did not take into account the finer features of spores, for future similar studies, experiments could be targeted at the evaluation of effect of size, weight and bio-volume of spores on the amounts of 16:1ω5c detected particularly in relation to the stage of spore development. This will result in more accurate estimates of infectivity.

The total amount of FAMEs extracted from the spores of *Gi. margarita* was six times greater than for any of the other AM species studied (Table 5.1) but the amount of 16:1ω5c was much less (5% of the total FAMEs) compared to the spores of *G. coronatum* (62%), *G. mosseae* (50%) and *S. calospora* (50%). The spores of *Gi. margarita* weighed slightly more than the spores of the *Glomus* spp., but were only half the weight of the *S. calospora* spores (Figure 5.2a). The apparent difference in the amount of extractable FAMEs from the spores is thus not related to spore weight and suggests that the spores of some AM fungi (such as *Gi. margarita*) contain larger amounts of lipid per unit volume than the spores of other species. The larger amount of total FAMEs in the *Gi. margarita* spores appeared to be associated with high amounts of 18-C and 20-C fatty acids.

The amounts of 16:1ω5c in the hyphae of the different AM fungi was not directly measured in this project, but could be calculated by subtracting the amounts of 16:1ω5c contained in the estimated number of spores from the total amounts of 16:1ω5c detected in a soil sample (section 4.5.2, Chapter 4). Biomass estimations of AM hyphae in soil have been made by several researchers using the amount of 16:1ω5c detected by PLFA analysis (Olsson *et al.* 1995, 1998), in experimental systems using the amount of 16:1ω5c detected by NLFA analysis (Olsson & Wilhelmsson 2000) and inside the roots using total lipids, PLFA and NLFA for a root pathogen *Aphanomyces euteiches* (Larsen *et al.* 2000). The present study has also shown that the amount of 16:1ω5c detected in the FAME profiles of the spores of AM fungi can be used to measure the biomass of AM fungi in soil, both in the experimental systems or in field soils.

7.5 Comparing Microscopic Techniques with Fatty Acid Analysis

Agreement between traditional methods of detection and quantification of AM fungi and detection and quantification based on fatty acid analysis is central to realising the benefits of applying fatty acid analysis to the study of AM fungi. Therefore, correlating measurements based on traditional methods (spore counts and % root colonisation) with fatty acid analysis was central to this project. Correlations between % root colonisation of roots and the amount of 16:1ω5c detected in FAME profiles of the roots and soil and PLFA profiles of roots, were found to be positive with R^2 values ranging from 0.42-0.65 (Figure 3.3a, b, and c,

Chapter 3). Fatty acid 16:1ω5c was unique in being the only fatty acid showing a positive correlation with these parameters. The relationship was also positive but comparatively weaker between the amount of 16:1ω5c detected in the FAME profiles of colonised roots and the number of AM fungal spores in soil (Figure 3.3d).

A positive correlation between the traditional methods and FAME analysis was further confirmed in the study of 3 field soil samples which was set up specifically to compare the capacity of the respective methods to estimate AM infectivity in the soil (Chapter 6). Higher amounts of 16:1ω5c were consistently associated with higher values for % root colonisation and spore number in the pasture soil and in soil under clover plants. Regression analysis between amount of the 16:1ω5c detected in the soil and % root colonisation of four host plants grown in the soils showed a positive correlation with R^2 values ranging from 0.57-0.98 across the four hosts (Figure 6.3, Chapter 6). The amounts of 16:1ω5c were also positively correlated (R^2 = 0.79-0.85), with the number of spores in soil, confirming earlier results (Chapter 3).

7.6 Conclusions

The results of this project have confirmed the use of fatty acid analysis as a technique for studying AM fungi. The association of specific fatty acids with five different species of AM fungi have clearly presented the option of using fatty acids for the detection of AM fungi in soil or colonised roots, differentiation between species of different AM fungal genera and specific identification of some AM species especially *Gigaspora* spp. Fatty acid 16:1ω5c has been confirmed as a general biomarker for AM fungi and as a potential tool to quantify AM fungi in soil and plant roots. Positive correlations were found between traditional methods for detection and quantification of AM fungi (ie. spore counts, % colonisation of plant roots) and the amount of 16:1ω5c detected in FAME profiles of soil (including field soils) and plant roots. In addition, measurement of the amount of 16:1ω5c in the spores of different AM fungi provides a basis for determining what proportion of the 16:1ω5c detected in soil samples can be attributed to spores (if their number is measured) and what proportion can be attributed to fungal hyphae.

The amounts of 16:1ω5c in AM propagules could be used for the measurement of biomass of propagules where the traditional techniques are difficult to apply eg. AM population estimations in the field. The labour-oriented spore counting can be easily replaced with the quicker FAME analysis of spores. Similarly, a quicker FAME analysis of colonised roots can be used to give estimated AM biomass in terms of amounts of 16:1ω5c, to replace the hours of microscope use in calculating estimated % colonisation. One of the biggest drawbacks of using spore counting from a field sample is that a sizeable population of spores can be lost during the wet sieving, which could be avoided when using FAME analysis. Another benefit of using soil FAME for biomass estimations is that the soil gives a holistic count of all the fungal propagules, including those attached permanently with the rootlets, which is not possible with the traditional methods where essentially some sample is lost. Also, the same sample can not be used for spore as well as hyphae measurements by traditional method whereas it is possible with the FAME technique.

Like other methods FAME analysis also has its limitations, and caution needs to be exercised when applying the technique to soil samples where low background levels of 16:1ω5c from other microorganisms may be present. This is probably of no concern with plant roots as this fatty acid was not detected in the non-mycorrhizal roots of the host plants tested in this work. In addition, FAME analysis has been found to have limited applicability in identification of AM fungi at or below the genus level.

7.7 Future Work

In future work, FAME analysis should be extended to a larger range of AM fungi including the species of all genera of order Glomales, so that a comprehensive data set of profiles can be prepared. Such a data set would be potentially useful for the identification of species of AM fungi, similar to the application of fatty acid analysis used for bacterial identification (Microbial I. D. Inc., Newark, DE).

Use of fatty acid techniques to detect AM fungi in a variety of host species should be investigated to assess the influence of host species on fatty acid profiles of AM species.

Recent reports have strongly suggested that AM fungi interact directly with root pathogens and reduce the level of energy reserves available to them and hence sporulation (Larsen & Bodker 2001; Graham 2001). There is also an indication of competition for organic carbon and possibly other nutrients between the two types of root-infecting fungi (Peng *et al.* 1993). In future studies, the qualitative and quantitative analyses of marker fatty acids can be used as indicator of energy status of the interacting fungi and the host. For this to occur, it is again important to link specific fatty acids to specific AM species.

The validity of fatty acid technique for identification and differentiation at or below species level could only be tested with a few species of *Gigaspora*, which was not sufficient to draw conclusions in this project. In future, a large number of *Gigaspora* species could be studied to confirm the potential use of fatty acids 20:1ω9c and 18:1ω9c in identification of *Gigaspora* species. Similar specific fatty acids could also be identified with other major AM fungal species by selecting large number of isolates of each species over a range of families of Glomales. This kind of extensive database thus compiled could be comparable to the one that is currently being used for bacteria.

Although 16:1ω5c has been found to be a useful marker for AM fungi in field soils, more work is required to validate the use of fatty acid 16:1ω5c in field soils for both detection and quantification of AM fungi. Also, more information is required concerning the dynamics of 16:1ω5c in the various structures associated with AM fungi and especially in relation to spore age and development and differences between spores and vegetative structures. Such parameters will be helpful in accurate measurement of the biomass of AM fungi in field soils and thus will be helpful in assessing the general health of soil for optimum agricultural use.

APPENDIX :

I. Solutions for FAME & PLFA:

FAME:

Saponification Reagent (Reagent 1)	45g NaOH 150ml Methanol 150ml RO Water Add NaOH pellets to water and methanol keep stirring, until pellets are dissolved
Methylation Reagent (Reagent 2)	325ml 6.0N HCl 275ml Methanol Add acid to methanol whilst stirring
Extraction Solvent (Reagent 3)	200ml Hexane 200ml Methyl-t-butyl-ether (MTBE) Add MTBE to hexane and stir
Base Wash (Reagent 4)	10.8g NaOH 900ml RO Water Dissolve pallets in water

PLFA:

1-Phase Buffer	Chloroform: Methanol: Phosphate Buffer (1:2:0.8 v/v/v) Mixed vigorously, heat let out
Phosphate Buffer	1M K_2HPO_4 (pH 7.4) 8.7g of K_2HPO_4 dissolved in 1000ml MilliQ Water. pH adjusted with 1M HCl
Methanol: Toluene	1:1 (v/v)
0.2M KOH water	11.22g flakes of KOH dissolved in 1000ml
Acetic Acid	1N CH_3COOH

II. Solutions for Plants and Staining

Long-Ashton Nutrient Solution (For Leeks)	4ml/L 2mM K_2SO_4 4ml/L 1.5mMMg$SO_4.7H_2O$ 4ml/L 4mM $CaCl_2.2H_2O$ 2ml/L 4mM $(NH_4)_2SO_4$ 4ml/L 8mM NaNO3 1ml/L FeEDTA (4g/100ml)
Standard Phosphorus Solution	Dissolve 4.3937g KH_2PO_4 in 1L RO water
Sodium Hypo-chlorite Solution	Dilute HTP pool chlorine ™ 1:2 in RO water
Trypan Blue (0.05%)	600ml Lactoglycerol (for stains) 0.3g Trypan Blue Roots are stained for 20-40 min at room temp
Lactoglycerol (for stains)	Lactic Acid 650ml Glycerol 600ml RO Water 800ml

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