THE ROLE OF ARBUSCULAR MYCORRHIZAL FUNGI IN THE BIOTRANSFORMATION OF COAL AND APPLICATION IN DUMP REHABILITATION

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ABSTRACT

Fundamental processes underpinning the biotransformation of coal by fungal biocatalysts have been intensively investigated, however, limited large-scale industrial applications using such systems have been reported. The un-anticipated sporadic growth of *Cynodon dactylon* on the surface of un-rehabilitated discard coal dumps has been noted and this was found to be coupled with the breakdown of coal into a humic soil-like material in the top 1.5 metres of the dumps. Extensive fungal growth was observed to be associated with the *Cynodon dactylon* root system and examination of plant roots indicated the presence of mycorrhizal fungi.

Analysis of the *Cynodon dactylon* plant roots around which coal biotransformation was occurring confirmed the presence of arbuscular mycorrhizal colonisation with the species *Glomus clarum*, *Paraglomus occultum*, *Gigaspora gigantea* and *Glomus mosseae* identified to be associated with the plants. Further molecular characterisation of non-mycorrhizal rhizospheric fungi showed the presence of fungal species with coal-degrading capabilities that most likely played a role in the coal biotransformation observed. The discard coal dump environment was simulated in pot and column studies and coal biotransformation was reproduced, with this process enhanced by the addition of mycorrhizal and non-mycorrhizal rhizospheric fungal inocula to the environment. Mycorrhizal and non-mycorrhizal species in the inoculum were re-isolated from the simulated environment fulfilling a number of Koch's postulates and indicating a causal role in the biotransformation of coal. An inversion of conventional mycorrhizal colonisation was demonstrated in this system with reduction in extraradicular presence and an increase in intracellular colonisation compared to soil controls.

A descriptive model was formulated suggesting a two-part fungal system involving organic carbon and nutrient exchange between the plant, mycorrhizal fungi and non-mycorrhizal coal-degrading rhizospheric fungi ultimately resulting in the biotransformation of coal. The biotransformation observed was comparable to reports of "rock-eating fungi". Results suggest that the biological degradation of coal *in situ* with the production of a soil-like substrate could provide a feasible method of discard coal dump rehabilitation as well as provide a humic-rich substrate that can be utilised in further industrial applications.

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ABBREVIATIONS

ATP - Adenosine Triphosphate
ANOVA - Analysis of Variance
~ - Approximately
AMF - Arbuscular Mycorrhizal Fungi
BLAST - Basic Local Alignment Search Tool
BSA - Bovine Serum Albumin
cm - Centimetre
cm^2 - Centimetre Cubed
°C - Degrees Celsius
dNTP - Deoxynucleoside triphosphate
DNA - Deoxyribonucleic Acid
ECCN84 - EBRU Culture Collection Number 84
EBRU - Environmental Biotechnology Research Unit
ELISA - Enzyme-Linked Immunosorbent Assay
EDTA - Ethylene Diamine Tetra-Acetic Acid
GC - Gas Chromatography
g - Grams
ha - Hectare
ICP - Inductively Coupled Plasma
INVAM - International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi
ITS - Internally Transcribed Spacer
IPTG - Isopropyl β-D-1-thiogalactopyranoside
kg - Kilogram
kPA - Kilopascals
L - Litre
LMW - Low Molecular Weight
MS - Mass Spectrometry
mRNA - Messenger Ribonucleic Acid
MT - Metallothioneins
m - Metre
μl - Microlitre
μm - Micrometre
mg - Milligram
ml - Millilitre
mm - Millimetre
mM - Millimolar
mya - Million Years Ago
M - Molar
MPN - Most Probable Number
NCBI - National Centre for Biotechnology Information
OD - Optical Density
ppm - Parts Per Million
/ - Per
% - Percent
PC - Phytochelatin

PCR	-	Polymerase Chain Reaction
PLVG	-	Polyvinyl–Lactic acid–Glycerine
rpm	-	Revolutions Per Minute
RNA	-	Ribonucleic Acid
r	-	Ribosomal
SEM	-	Scanning Electron Microscopy
SOC	-	Super Optimal Catabolite Repression
SOD	-	Superoxide dismutase
Taq	-	*Thermus aquaticus*
T	-	Time
X	-	Times
t	-	Tonnes
TOC	-	Total Organic Carbon
TAE	-	Tris-Acetate-EDTA
V	-	Volts
v	-	Volume
X-gal	-	5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside

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CHAPTER 1:

MYCORRHIZAL FUNGI AND MINE REHABILITATION

1.1 Mine Closure and Rehabilitation

In recent years, the licensed closure of mining operations has emerged as a major liability for mining companies and involves environmental, social, financial and safety policy issues (World Bank, 2002). Mine closure in South Africa is governed by the Mineral and Petroleum Resources Development Act (Act 28 of 2002). Amongst its many objectives, the Act clearly states that "holders of mining and production rights must contribute towards the rigorous improvement of both biophysical and socio-economic impacts that may result in areas in which they are operating". Ghose (2004) noted that in implementing these objectives, affected land must be returned to pre-mining productivity with rehabilitation providing an "essential part of developing mineral resources in accordance with the principles of ecologically sustainable development" (Ghose, 2005). The rapidity with which post-mining steps are implemented is therefore a key factor where rehabilitation contributes towards sustainable development in the mining area (Hoadley *et al.*, 2002).

The basic principles of mine rehabilitation have been extensively reviewed (Lubke and Avis, 1999; Cooke and Johnson, 2002) with a number of basic steps required for efficient mine closure. It is essential that rehabilitation strategies are devised prior to the initiation of mining operations that will ensure post-mining land use which is compatible with the climate, soils and topography of the final land form. Potentially toxic products of mining that could impact the post-mining environment must be identified prior to mining and topsoil overlying mineral seams must be characterised and retained for rehabilitation immediately after mining ceases. Rehabilitation must also be a progressive process with the rate of implementation keeping pace with the rate of mining, where possible. In this process, drainage patterns must be retained and cleared vegetation spread on disturbed lands. Finally, on conclusion of the process, rehabilitated land must be monitored and managed until such time as the vegetation is self-sustaining.

1.2 Rehabilitation of Discard Coal Dumps

Mine waste discard, produced during mining operations, is a term that refers to initially mined product that on analysis is judged not to be of commercially viable grade (Maree *et al*., 2004b). In the coal mining industry, water is generally used to grade coal into different particle size fractions with waste material often stock-piled in waste discard dumps. However, in coal mining, dumps are not only generated by waste discard but also by coal spoils. Coal spoil dumps are formed mainly by the stock-piling of overburden material consisting of soil, rock and lower grade coal which lies above the coal seams (Carvey *et al*., 1977; Harrison, 1978).

Discard dumps are rehabilitated in a variety of ways but mainly the application of a layer of clay and topsoil is utilised to establish plant communities and generally involves three steps. Dumps are firstly graded to shape them into a stable configuration. The dumps are then covered with an impermeable material such as clay to prevent ingress of rain and oxygen into dumps which can contribute to oxidation and the production of sulfuric acids from sulphides as well as spontaneous combustion (Cohn *et al*., 2006). Compaction when laying down the dumps is utilised to prevent oxygen ingress and lime is added to neutralise any acid-mine drainage that might form (Bell *et al*., 2001). The clay layer is covered with soil and vegetated to help stabilise the cladding material. A key problem, however, is that in the process of removing the desired mineral, especially in open-cast mining, soils originally removed from the mining site are dissipated, are buried or due to stock-piling are eventually not viable for use as topsoil once such rehabilitation commences (Kundu and Ghose, 1997a; Kundu and Ghose, 1997b).

Garner *et al.* (2000) reported a case study on the rehabilitation of discard dumps formed at the Springbok Valley Colliery (Mpumalanga, South Africa) that illustrates several aspects of these rehabilitation principles. Highlighted were the costs, both in time and material, which are involved in this undertaking. They applied 3tonnes/hectare (t/ha) lime onto the discard dump and then 250 millimetre (mm) subsoil followed by a capping of 300mm topsoil. An irrigation system was set up on site with water delivered as a fine mist to prevent erosion through runoff. Superphosphate fertilizer (1t/ha) was spread and a mixture of *Cenchrus ciliarus* (blue buffalo grass), *Chloris gayana* (Rhodes grass) and *Cynodon dactylon* (Bermuda grass)

(*C.dactylon*) planted. The site was then irrigated for several months and fertilised repeatedly.

1.3 Phytorehabilitation of Discard Dumps

The establishment of vegetative covers on cladded coal mine discard is a challenging task mainly due to problems associated with mine spoils such as compaction, poor water-holding capacities, poor infertility, high acidity or salinity and extreme temperatures generated (Dutta and Agrawal, 2003). These dump substrates are often physically, nutritionally and biologically poor in nature and devoid of beneficial microorganisms (Mehrotra, 1998). However, Bradshaw (1997) noted that dump cladding materials can develop over time into fully functional soils. Although initial problems in the establishment of plant growth may occur, once established, plants will contribute to the build up of organic matter, the lowering of soil bulk density and bringing mineral nutrients to the surface enabling accumulation in an available form. Some plant species also fix and accumulate nitrogen (N) in sufficient quantities to support a functionally diverse ecosystem. Establishment of natural plant succession on such dumps has been documented, although this will often take a long time to occur (Jha and Singh, 1992).

1.4 Mycorrhizal Fungi and Phytorehabilitation of Dumps

The symbiotic relationship between plant roots and mycorrhizal fungi has been studied by biologists for many generations and in the late 1880s these associations were given the name mycorrhizas, a term derived from Greek for “fungus-root” (Harrison, 1999). This symbiotic association alters interactions between the plant and soil thereby enhancing plant growth under potentially stressful environmental conditions (Smith and Read, 1997). As a result, considerable interest has been focussed on the possible utilisation of mycorrhizal fungi in the reclamation of mine discards and spoils. Much of this interest has stemmed from experimental work that demonstrated that mycorrhizal fungi are able to improve the survival and growth of seedlings by alleviating most of the deficiencies that plants encounter during their establishment on such mine wastes. Initial studies on coal mining wastes were conducted by Schramm (1966), who when investigating plant colonisation of anthracite wastes in Pennsylvania, concluded that early ectomycorrhizal development was essential for seedling establishment of *Betula lenta* (sweet birch)*, Betula populifolia* (gray birch)*, Pinus rigida* (pitch pine)*, Pinus virginiana* (Virginia pine)*, Populus tremuloides* (quaking aspen)*, Quercus rubra* (northern red oak) and *Quercus velutina* (black oak) which were found to be able to grow on this waste material. The plants colonising this bare and predominantly N deficient waste were either N-fixing plants or ectomycorrhizal plant species, with the ectomycorrhizal plants proliferating. Seedlings from either wind-blown or artificially planted seed of these plant species, that did not establish an ectomycorrhizal association, soon became chlorotic and died. The ectomycorrhizal fungal species identified in association with healthy plants in this environment were *Inocybe lacera, Thelephora terrestris, Pisolithus tinctorius, Amanita rubescens,* and *Scleroderma aurantium.*

An early study involving endomycorrhizal fungi was conducted by Daft and Nicolson (1974) who found an abundance of endomycorrhizal fungi associated with the roots of a variety of herbaceous plants, including grasses. These plants were growing in anthracite and bituminous coal wastes in Pennsylvania and bituminous coal wastes in Scotland. Spores of *Gigaspora gigantea* (*Gi.gigantea*) were identified and collected from the Pennsylvania wastes with the mycorrhizal fungus found to enhance the growth of colonised corn plants in coal waste in comparison to un-colonised plants. It was concluded that endomycorrhizal fungi may be essential for the survival and growth of the herbaceous plants on coal wastes.

Subsequent studies have demonstrated similar results. Call and Davies (1988) inoculated seedlings of *Bouteloua curtipendula* (side-oats gama), *Sorghastrum nutans* (Indiangrass) and *Panicum coloratura* (kleingrass) with the mycorrhizal fungal species *Glomus fasciculatum* and *Gigaspora margarita* (*Gi.margarita*)*.* Inoculation was carried out in sterile containers and after germination, plants were transplanted into mycorrhizal-free lignite discard in the Post Oak Savannah region of Texas*.* After three growing seasons it was observed that plants inoculated with these arbuscular mycorrhizal fungi (AMF), a group of endomycorrhizal fungi, showed greater survival percentages and a better below and above-ground biomass than un-inoculated plants. Inoculated plants also showed higher levels of N and phosphorus (P) in their above-ground biomass compared to control plants. It was thus concluded that the mycorrhizal fungi associated with the plants enhanced the survival and growth of the

3 grass species by making effective use of the limited nutritional resources available in lignite discard. Similarly, Kahn (1981) investigated the effects of the AMF species *Glomus macrocarpus*, *Glomus mosseae* (*G.mosseae*) and *Sclerocytis rubiformis* on the growth of onions in un-sterilised coal washery waste. Mycorrhizal onions were significantly larger than non-mycorrhizal controls when biomass measurements were recorded. A wide range of subsequent studies have demonstrated the ability of AMF to enhance plant community establishment on potentially phytotoxic treated and un-treated mine wastes (Bi *et al*., 2003; Caproni *et al*., 2003; Ganesan *et al.,* 2004; Gaur and Adholeya, 2004; Da Silva *et al*., 2005).

1.5 Mycorrhizal Fungi

1.5.1 Mycorrhizas

Mycorrhizas are highly evolved mutualistic associations between soil fungi and plant roots. The partners in this association are members of the division Eumycota and most vascular plants (Harley and Smith, 1983; Brundrett, 1991; Kendrick, 1992). In mycorrhizal literature, the term symbioses is often used to describe these highly interdependent mutualistic relationships where the host plant receives mineral nutrients obtained from the soil by the fungus while the fungus obtains photosynthetically derived carbon (C) compounds from the plant (Harley and Smith, 1983). At least seven different types of mycorrhizal associations have been recognised, with associations involving different groups of fungi and host plants as well as distinct morphological features (Brundrett *et al*., 1996). Examples of these are arbuscular mycorrhizas, ericoid mycorrhizas, orchid mycorrhizas and ectomycorrhizas. Generally, mycorrhizal fungi can be divided into two groups: endomycorrhizal fungi and ectomycorrhizal fungi based on the part of the root they colonise (Smith and Read, 1997).

1.5.2 Arbuscular Mycorrhizal Fungi

Recent observations of fossil plants from the Devonian era suggest that one type of mycorrhizal association, the arbuscular mycorrhiza, has existed since approximately 400 million years ago (mya) thus indicating that plants have formed associations with AMF from the first colonisation of land (Pirozynski and Malloch, 1975; Remy *et al*., 1994). Today, arbuscular mycorrhizas remain the most common type of mycorrhizal association and exist in numerous ecosystems around the world (Harley and Smith, 1983; Allen, 1996; Smith and Read, 1997). In arbuscular mycorrhizal associations, Glomeromycota fungi produce arbuscules, hyphae and vesicles within root cortical cells with this colonisation being typical of endomycorrhizal fungi. Arbuscules greatly increase the surface area through which nutrient and C exchange between the plant and fungus can take place. Vesicles act as nutrient stores and extraradical hyphae extend out into the soil substrate and enhance nutrient uptake, primarily of P (Brundrett *et al*., 1996). Similar advantages are conferred to the plant in ectomycorrhizal associations. However, in these associations, a mantle (layers of fungal hyphae covering the root surface) forms around roots and a Hartig net (a tangled branching of fungal cells in a layer) is established between root cells (Brundrett *et al*., 1996). The Hartig net is considered to be the major site of nutrient exchange between the fungus and host plant.

Apart from enhanced nutrient uptake from soil, other advantages of mycorrhizal associations include improved soil structure and aeration, improved tolerance to stressful conditions such as drought and heavy metals and an increased tolerance to diseases and plant pathogens (Sutton and Sheppard, 1976; Graham and Menge, 1982; Allen and Allen, 1986; Hooker *et al.,* 1994; Azcon-Aguilar & Barea, 1996).

1.5.3 Development of Arbuscular Mycorrhizal symbioses

Genetic analysis has confirmed that AMF are asexual and reproduce clonally (Rosendahl and Taylor, 1997). These fungi produce large resting spores that are unusual in that they are multi-nucleate and, depending on species, can contain thousands of nuclei per spore (Becard and Pfeffer, 1993). Germination of AM fungal spores, and the initial growth of hyphal germ tubes, can occur even in the absence of a plant root. However, both root exudates and volatiles such as CO_2 can stimulate both of these processes (Becard and Piche, 1989**;** Gianinazzi-Pearson *et al*., 1989; Becard *et al*., 1992; Chabot *et al*., 1992; Balaji *et al*., 1995). In some cases, as hyphae approach plant roots, extensive branching has been observed in response to exudates in the vicinity of plant roots (Giovannetti *et al*., 1993b; Giovannetti *et al*., 1994). This response has not been observed when hyphae encounter non-host plants, suggesting that recognition between the plant and AMF does occur. Non-recognition could be due to lack of an appropriate signal, but could also include the production of inhibitory compounds produced by the plant (Schreiner and Koide, 1992).

The symbioses between the host plant and AM fungus is initiated when the fungal hyphae contacts the root surface. Differentiation takes place and the formation of an appressorium, a flattened disk-like hyphal organ that aids penetration, occurs. Unlike the stimulation of hyphal branching and growth by root exudates, appressorium development only takes place in the presence of a plant root (Giovannetti *et al*., 1993a). It has been demonstrated that *Gi.margarita* forms appresoria *in vitro* on purified epidermal cell walls from carrot roots, a host, but not on cell walls isolated from sugar beet, a non-host (Nagahashi and Douds, 1997). In this case, the fungus was also able to differentiate between cortical, vascular and epidermal cell walls, only forming appressoria in the presence of epidermal cell walls.

Development of penetration hyphae occurs after appressorium formation which facilitates entry into the plant root. This occurs in a variety of ways with some species' hyphae entering plant roots by forcing their way between epidermal cells, whereas in other cases, hyphae penetrate an epidermal or root hair cell wall and grow through the cell (Bonfante-Fasolo, 1984). The exact mechanisms involved in penetration of root cells are unknown but in comparison to a number of fungal plant pathogens it has been suggested that the specific and localised production of cell wall-degrading enzymes, in conjunction with mechanical force, facilitates hyphal entry (Bonfante-Fasolo and Perotto, 1995). However in the case of AMF, this appears to be done without eliciting an immune response by the plant. AMF produce exo- and endoglucanases, cellulases, xyloglucanases, and pectolytic enzymes including polygalacturonase, all of which, as 'digestive enzymes', would accelerate their passage through a plant cell wall (Garcia-Romera *et al*., 1991; Garcia-Garrido *et al*., 1992; Garcia-Garrido *et al*., 1996; Rejon-Palomares *et al*., 1996). In studies where purified epidermal cell wall fragments were used to examine appressoria development, viable penetration hyphae did not form and penetrate the wall, thereby indicating that processes subsequent to appressorium formation most likely require intact cells (Nagahashi and Douds, 1997). Plant mutant studies where AM fungi are able to form appressoria but cannot develop further, suggest that the plant controls this development step (Harrison, 1999).

Following entry into the root, the internal development of the fungus appears to be influenced by the plant being colonised with a single species of fungus showing significantly different morphological growth patterns (Gerdemann, 1965; Jacquelinet-Jeanmougin and Gianinazzi-Pearson, 1983). The two main AM fungal colonisation patterns observed are *Paris* and *Arum* types, and are named after the plant species in which they were first described (Smith and Smith, 1997). In *Arum*-type colonisation, which is often regarded as a "typical arbuscular mycorrhizal colonisation" and is frequently described in fast growing systems, penetration of the root is initially followed by intercellular hyphal growth. However, in some cases the fungus will penetrate the root exodermis and form hyphal coils in the exodermal cells as it passes through (Bonfante-Fasolo, 1984; Smith and Read, 1997). On reaching the inner cortex of the root, branching arising from the intercellular hyphae occurs, this penetrates the cortical cell walls and differentiates terminally within the cell to form dichotomously branched structures known as arbuscules (Harrison, 1999).

Though arbuscules develop within cells, they remain essentially apoplastic as the plant plasma membrane extends to completely surround it. The fungal cell wall becomes progressively thinner with time as the arbuscule develops and, as a result, there is a wide intracellular interface in which the plant and fungus are in close contact, separated only by their membranes and a narrow plant-derived apoplast (Smith and Gianinazzi-Pearson, 1988; Bonfante-Fasolo and Perotto, 1990; Bonfante-Fasolo and Perotto, 1992). It is thought that it is at this interface that P and C are transferred between symbionts, although some speculate that the intercellular hyphae might be responsible for C uptake (Smith and Smith, 1989; Smith and Smith, 1990). Despite the intensive effort put into the development of arbuscules by both plant and fungus, the life span of an arbuscule is only a few days. After formation it collapses and decays leaving the cell undamaged and ready to host another arbuscule (Alexander *et al.*, 1988; Alexander *et al.*, 1989). Following formation of arbuscules, some species of AM fungi also form lipid-filled vesicles within the roots, which are presumed to act as a storage reserve for the fungus (Smith and Gianinazzi-Pearson, 1988).

In *Paris*-type colonisation, colonisation of the roots is characterised by the extensive development of intracellular coiled hyphae which spread directly within the cortex from cell to cell. Arbuscules can grow from these coils and there is very little intercellular growth, a consequence of which is that the rate of growth of the infection units within the root is much slower than that of the *Arum* type (Smith and Read, 1997). Also often observed is what can be termed intermediate colonisation types, which are characterised by features from both *Arum* and *Paris* type colonisation (Skinner, 2006).

On completion of root cortex colonisation, AM fungal hyphae develop expansively within the soil with external mycelia playing a pivotal role in the mycorrhizal symbiosis. Their functions include the acquisition and subsequent translocation of mineral nutrients from the soil to the plant, the exudation of C products of photosynthesis into the rhizosphere to aid nutrient acquisition, colonisation of additional roots and the production of spores (Harrison, 1999). In addition to their role in the symbiosis, the extra-radical hyphae contribute to soil stability by the aggregation of soil particles, mediated in part by glyco-proteins produced by the hyphae (Wright *et al*., 1996; Wright and Upadhyaya, 1996).

Although it is well known that C is transferred from the plant to the fungus, evidence on the form of C that is transported was initially lacking (Ho and Trappe, 1973; Bevege *et al*., 1975). *In vivo* ^{13}C nuclear magnetic resonance spectroscopy data strongly suggests that glucose is the form of C utilised by mycorrhizal fungi with such recordings further supported by studies of isolated arbuscules that have been observed to use glucose for respiration (Shachar-Hill *et al*., 1995; Solaiman and Saito, 1997). Although during mycorrhizal associations C allocation to the roots increases, the amounts of C estimated to escape out of intact root cells into the apoplast are thought to be insufficient to account for the amount of fungal growth occurring in mycorrhizal roots. Thus, enhanced efflux of C, or a decrease in the level of competing host uptake systems has been proposed (Patrick, 1989; Smith and Smith, 1989; Smith and Smith, 1990; Schwab *et al*., 1991). Phosphorus movement in the plant-fungus symbiosis involves a number of membrane transport steps, starting with the uptake of P across the external mycorrhizal hyphae (Harrison, 1999). This uptake is followed by translocation back to the internal fungal structures where it is thought to be released from the fungus across the arbuscule membrane, and then taken up into the plant by transporters on the peri-arbuscular membrane (Smith and Gianinazzi-Pearson, 1988; Smith and Smith, 1989; Smith and Smith, 1990).

1.6 Mycorrhizal Weathering of Minerals

A critical initial study investigating the weathering ability of mycorrhizal fungi was conducted by Jongmans *et al.* (1997) who investigated the network of numerous tubular pores found associated with weatherable minerals under a variety of European coniferous forests, pores formed by organic acids exuded by fungi. The study confirmed that the mineral weathering observed was due to symbiotic mycorrhizal hyphae that translocated dissolved minerals from these micropores to the host plant. As this process would bypass competition for nutrient uptake by other organisms, the pathway discovered challenged many ideas present at the time describing nutrient uptake from bulk soil and introduced the term “rock-eating fungi”.

Physical and chemical weathering of the primary rock of the earth results in the release of dissolved mineral elements and residues into the biological environment (Landeweert e*t al*., 2001). It is believed that the rise of vascular land plants about 400mya led to vastly increased mineral weathering (Berner, 1992; Drever, 1994). Some of the products of photosynthesis formed in plant leaves are translocated down the length of the plant and end up as organic acids that are exuded by plant roots into the surrounding soil. Together with CO_2, which is excreted into the soil by root respiration and the heterotrophic respiration of soil microorganisms, such organic acids have been reported to greatly enhance the dissolution of primary silicate minerals thereby mobilizing essential lithophilic plant nutrients (Landeweert e*t al*., 2001). The C-rich root exudates of plants also support large communities of microorganisms such as rhizospheric bacteria and fungi that further accelerate weathering of minerals by further excreting organic acids, phenolic compounds, protons and siderophores (Drever and Vance, 1994; Illmer *et al*., 1995). Such plant-induced mineral weathering must have facilitated the subsequent spread of land plants by increasing the availability of essential plant nutrients. Such processes also produced secondary minerals such as clays and released iron (Fe) and aluminium (Al) oxides, the formation of which would provide soil material for anchorage and enhance water-holding properties (Landeweert *et al*., 2001).

Studies on mycorrhizal weathering of rocks and minerals have primarily been carried out on ectomycorrhizal fungi.

1.6.1 Organic Acids and Weathering

Soluble organic acids that affect mineral weathering in soils originate from a number of sources. Medium to high molecular weight organic acids, such as humic substances, are less effective in driving mineral dissolution than are low molecular weight (LMW) organic acids produced by plant roots and soil microorganisms (Ochs, 1996). Although they constitute only a minor fraction of the total organic acids in soil solution, owing to the acidifying and complexing properties they possess, LMW organic acids are generally considered to be the most important biological weathering agents in soils (Ochs, 1996; Barker *et al.*1998). Depending on the number and configuration of carboxylic and phenolic groups and the related acid strength, organic acids provide H^+ for protonation of the mineral surface and chelate cations (Welch and Ullman, 1993). Concentrations of LMW organic acids in the bulk soil solution are generally too low to accelerate mineral weathering. However concentrations in the microenvironments around microbes, fungal hyphae and roots are often high enough to do so (Drever and Vance, 1994; Barker *et al*., 1998; Arocena *et al*., 1999; Banfield *et al*., 1999). Of the several LMW organic acids released by plant roots, bacteria and fungi, oxalate, citrate and malate are the strongest chelators of trivalent metals such as Al^{3+} and Fe^{3+} (Jones, 1998).

Oxalic acid has the highest acid strength and forms complexes with potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), zinc (Zn), copper (Cu), Al and Fe and is commonly produced in large quantities by a variety of fungal species (Dutton and Evans, 1996; Gadd, 1999). Although potentially toxic to fungi (Gadd, 1999), oxalic acid may play a number of functional roles including providing an electron donor in the degradation of ligno-cellulose (Dutton and Evans, 1996).

1.6.2 Mobilisation of Nutrients and Mineral Weathering by Ectomycorrhizal Fungi

The positive effects of ectomycorrhizal fungi on plant nutrition have conventionally been attributed to the quantitative effects of the extraradical mycelium on the uptake of dissolved nutrients from the soil and subsequent transport to the roots of host plants (Landeweert *et a*l., 2001). Subsequent attention has been paid to the qualitative effects of the symbiosis on plant nutrition and the ability of ectomycorrhizal fungi to use organic N and P sources which would otherwise be largely unavailable to plants (Smith and Read, 1997). Ectomycorrhizal fungi might also be able to access pools of P within mycelia of saprotrophic fungi (Lindahl *et al.*, 1999). Such types of nutrient uptake generally represent 'short cuts' to traditionally described pathways of decomposition and mineralization and highlight the important function of ectomycorrhizal fungi in ecosystem nutrient recycling.

When grown on agar plates, ectomycorrhizal fungal species have been shown to produce LMW organic acids and are able to solubilise Ca phosphates deposited on agar (Leyval and Berthelin, 1985; Lapeyrie *et al.*, 1991) as well as mobilise K^+, NH_4^+ and Ca^{2+} trapped inside mineral interlayer spaces (Paris *et al.*, 1995(a); Paris *et al.*, 1995(b); Paris *et al.*, 1996). The simultaneous depletion of K^+ and Mg^{2+} in the growing medium increased mineral weathering (Paris *et al.*, 1996). Weathering performance *in vitro* though can vary greatly between different ectomycorrhizal fungal species, between different strains belonging to the same fungal species and between monokaryotic and dikaryotic mycelia of the same ectomycorrhizal fungal species (Lapeyrie *et al.*, 1991).

The weathering of soil minerals *in vivo* has been observed for host tree seedlings growing in symbiosis with ectomycorrhizal fungi (Leyval and Berthelin, 1985; Olsson and Wallander, 1998; Wallander and Wickman, 1999; Wallander, 2000a; Wallander, 2000b). Phosphorus was observed to be mobilized from apatite and K mobilized from biotite by ectomycorrhizal pine seedlings in long-term pot experiments. In these experiments though, only traces of organic acids were detected in the rhizosphere of mycorrhizal pines and the release of essential plant nutrients was seldom positively correlated to the oxalic acid concentration in the soil solution. The high microspatial variability in concentrations, and rapid microbial consumption of C-rich plant and fungal exudates, obscures the relationships between organic acid concentrations and ectomycorrhizal effects on weathering, as well as nutrient uptake, under more natural conditions (Landeweert *et al.*, 2001). Dual inoculation with rhizobacteria and an ectomycorrhizal fungus has shown that exudation of organic acids by roots is indeed modified by the presence of ectomycorrhizal fungi as well as rhizobacteria (Leyval and Berthelin, 1985). Moreover, ectomycorrhizal fungal exudates, bacterial numbers and bacterial activity appear to change with the kind of mineral applied (Olsson and Wallander, 1998) thereby probably influencing mineral weathering effects. When grown in axenic conditions, ectomycorrhizal pine seedlings generally produce more oxalic acid than non-mycorrhizal seedlings (Ahonen-Jonnarth *et al.*, 2000).

1.6.3 Mineral Weathering by Individual Ectomycorrhizal Hyphae

Weathering of soil minerals is not only brought about by products of ectomycorrhizal root tips, but can also be enhanced by ectomycorrhizal hyphae that radiate outwards from the colonised root tips (Landeweert *et al.*, 2001). In soil, hyphae are able to tightly enclose mineral particles as well as penetrate mineral interlayer spaces (Robert and Berthelin, 1986; van Breemen *et al.*, 2000a) with the interior of weatherable minerals also potentially becoming exploited by mycorrhizal hyphae. Thin section micrographs of feldspars and hornblendes from coniferous forest soils have shown open tunnels with rounded ends and curved tracks (Jongmans *et al.*, 1997; van Breemen *et al.*, 2000b). It was noted that the tunnels with a constant diameter differed morphologically from crystallographically oriented etch pits and saw-tooth cracks that would result from chemical weathering (Lasaga and Blum, 1986). The presence of hyphae inside the tunnels indicated that single hyphae reach the interior of the tunnelled weatherable minerals (Jongmans *et al.*, 1997; Berner and Cochran, 1998; van Breemen *et al.*, 2000b).

Some feldspars contain apatite inclusions and ectomycorrhizal hyphae may be able to access these enclosed sources of P through the ‘tunnel growth’ described, thereby exploiting a mineral P source unavailable to plant roots (Rogers *et al.*, 1998). The selective dissolution of Ca-rich inclusions in volcanic glass has also been ascribed to acid excretion by invading plant symbiotic fungal hyphae (Berner and Cochran, 1998) and similarly, bacteria in groundwater systems have been shown to be able to selectively colonize and weather P-rich feldspars when P is in short supply (Rogers *et al*., 1998).

The process of mineral weathering results primarily in the formation of soil and therefore has important biogeochemical consequences. Mycorrhizal fungi have thus most likely contributed significantly to soil formation and global element cycling through the effects of mineral weathering and podzolization (Landeweert *et al*., 2001). However, mineral weathering can create a wide variety of challenges for the plant-mycorrhizal symbiosis as well, including the accumulation of potentially toxic heavy metals. Such phytotoxic metals would also be expected to be readily encountered in areas of mining activity.

1.7 Metal Pollution and Mycorrhizal Fungi

1.7.1 Metals in the Environment

Pollution of the biosphere with toxic metals as a result of man-made activities poses a major threat to environmental and human health (Leyval *et al.*, 1997). In addition to metals of geochemical origin that may at times reach high concentrations (Jeng and Bergseth, 1992), the sources of metals in soil are diverse and include the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments and the use of pigments and batteries (Leyval *et al.*, 1997). These metals are commonly called heavy metals, although this term strictly refers to metallic elements with a specific mass higher than $5 g/cm^2$ that are able to form sulphides (Adriano, 1986). The term trace metals would be more correct since the latter is based only on concentration, as for example, one would refer to ~0.1% in soil or 100 milligrams/kilogram (mg/kg) in dry matter of biological samples of a trace metal. However in environmental studies when describing metal pollution, the term heavy metal is generally used. Some of the metals such as Zn, Cu, Mn, nickel (Ni) and cobalt (Co) are micronutrients necessary for plant growth, while others are not readily required for biological function, such as cadmium (Cd), lead (Pb) and mercury (Hg) (Marschner, 1995). Heavy metals in this sense will be considered as toxic elements at high concentrations not readily required as micronutrients.

Metals in soil are present as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides. They may form part of the structure of silicate minerals indigenous to soil (Leyval *et al*., 1997). The toxicity of metals in soil depends on their bioavailability which can be defined as their ability to be transferred from a soil compartment to a living organism (Juste, 1988). According to Berthelin *et al.* (1995), metal bioavailability is a function not only of total concentration but also of physico-chemical factors such as pH and clay content as well as biological factors such as bioaccumulation and solubilisation. Various techniques are used to estimate the bioavailability of metals in soil. These include chemical extractions and biological tests using plants or micro-organisms (Sauerbeck and Stypereck, 1985). High metal concentrations in soil are toxic to bacteria and fungi. Metal tolerance in soil micro-organisms has been studied with regards to the removal of metals from polluted soils. These studies have also looked to provide a biological understanding of the adaptation of living organisms to extreme environments (Leyval *et al*., 1997).

Metal pollutants threaten ecosystem sustainability through the alteration of above ground and below ground community structure. This may result in severe loss of floristic diversity due to removal of the more sensitive components of the community (Meharg, 2003). As the health of plants and their mycorrhizal symbionts is intimately linked, so also to understand a plants response to pollutants, it is essential to understand the response of the fungal symbionts (Meharg & Cairney, 1999). The ability of mycorrhizal plants to enhance plant growth on contaminated soils has been demonstrated in a number of studies (Lux and Cumming, 2001; Gonzalez-Chavez *et al*., 2002a; Gonzalez-Chavez *et al*., 2002b; Martino *et al*., 2002). Meharg & Cairney (2000) highlighted the potential of mycorrhizas, particularly ectomycorrhizas, to remediate and remove pollutants from contaminated sites. Although the main focus of their study was on organic contaminants, and was based on the enzymatic capabilities of mycorrhizal fungi to transform a wide range of organic contaminants, they pointed out that remediation strategies were also reliant on the mycorrhiza being resilient to metal pollutants. Polluted sites often contain a complex range of contaminants, and therefore, fungi must be resilient to co-contaminants as well as the pollutants targeted for degradation (Meharg, 2003).

1.7.2 Pollutant Detoxification Mechanisms

A number of principle strategies are utilised by plants and mycorrhizal fungi to detoxify toxic metal cations. In mycorrhizas, the plant and the fungus may operate these strategies independently or they may act in unison (Meharg, 2003).

1.7.2.1 Avoidance

There is ample evidence that plants and microorganisms are able to prevent certain toxicants entering their cells (Meharg, 1993). This can be achieved through a range of biochemical adaptations such as: (a) suppression of toxic influx transporters; (b) enhanced efflux of toxicants; (c) release of pollutant complexing agents into the surrounding soil; (d) precipitation or binding of toxicants onto cell surfaces; and (e) release of extracellular enzymes that alter the toxicants speciation so that it is absorbed or converted into a non-toxic species (Meharg, 2003).

1.7.2.2 Compartmentalisation

In organisms where a compartmentalisation strategy is operated, the pollutant is translocated to specific organs in the plant or fungus or to sub-cellular compartments where it is relatively non-toxic. The translocation of pollutants to specific organs/organelles is usually due to these organs having limited metabolic function and the sub-cellular compartments are invariably vacuoles (Meharg, 1993; Meharg, 1994). The compound that enters the cell may be altered to another form for either transport within the plant, or for the purpose of storage. For example, it has been postulated that metal chelators such as dicarboxylic organic acids, amino acids, phytochelatins (PCs) and metallothioneins (MTs) play a role in the transport and storage of metal ions (Meharg, 1994). Thus metal ion dynamics within the soil and cell are complex and the understanding of pollutant speciation in a cell-soil system is still limited.

1.7.2.3 Homeostasis

Metals such as Fe, Zn and Mn are also required as essential nutrients in limited concentrations. Plants and fungi must obtain these elements from soil or, in the case of non-essential elements, inadvertently take them up from soil. To acquire micronutrients that are also toxic in excessive concentrations, plants and fungi have evolved varying mechanisms to maintain cellular homeostasis including the detoxification of excess metals and also repair mechanisms to counteract damage caused by these metals (Meharg, 1994).

Homeostasis may be achieved by altering transport mechanisms that take up metal ions. Many nutrient transporters are under tight physiological regulation and this is often dependant on the nutrient status of the plant (Marschner, 1995). PCs and MTs are synthesised on plant and fungal exposure to a range of metals, complexing these metals and effectively detoxifying them (Cobbett, 2000; Cobbett & Goldsburough, 2002). Protein metal complex transporters and PCs in metal complexes, usually present in the tonoplast (the cytoplasmic membrane that surrounds the vacuole of the plant cell), have been identified, with the putative role of maintaining cytoplasmic toxic metal ion concentrations by transporting toxic metal ions into the vacuoles (Ortiz *et al.,* 1992; Ortiz *et al.,* 1995; Cobbett & Goldsburough, 2002). Studies demonstrating the use of mutants deficient in enzyme systems used by plants and fungi to regulate metal homeostasis have shown how essential these enzyme systems are in maintaining cell function when exposed to metals. For a mycorrhizal system, this was shown when an MT gene isolated from *Gi.margarita* (*GmarMT1*) was cloned into a hypersensitive *Schizosaccharomyces pombe* yeast strain, enhancing Cd and Cu insensitivity of this strain (Lanfranco *et al*., 2002). However Meharg (1994) noted that all physiological studies on plant or fungal populations adapted to survive on contaminated sites have failed to find a role for these enzymes in adaptive tolerance, although their presence is required for the adaptive mechanisms to work.

1.7.2.4 Molecular Analysis

Investigations into metal tolerance have not yielded a strong physiological basis for adaptive tolerances in plants or mycorrhizal fungi (Meharg, 2003). With the development of powerful molecular techniques, such as differential display, a method for the analysis of differential gene expression at the level of messenger ribonucleic acids (RNA), it is now possible to look at gene expression induced under stressed and non-stressed conditions. It is also possible to compare gene expression between sensitive and insensitive ecotypes, strains characterised by the ecological surroundings they inhabit. However, to obtain meaningful results, such experiments must be conducted under carefully defined conditions (Meharg, 1994). Studies have demonstrated that toxic metal ions do not just affect genes involved directly in metal sensitivity, the toxic stress they impose on cells will lead to general cellular disruption, which may differ between sensitive plant or fungal genotypes, and thus to a wide range of differential protein expression between these genotypes (Lanfranco *et al.,* 2002; Meharg, 2003; Repetto *et al*., 2003).

It appears that the most appropriate way of comparing gene or protein expression, or even enzyme activity, is the approach developed by Schat & Kalff (1992) who invested the role of PCs in Cu tolerance of *Silene vulgaris* (*S.vulgaris*). Tolerant and non-tolerant ecotypes of *S. vulgaris* were selected and a dose response study was conducted showing root extension against Cu concentration in the plants growth solution. Using dose response curves generated, they then chose a concentration for the tolerant plant and one for the non-tolerant plants that caused the equivalent amount of root inhibition in the two genotypes (i.e. concentrations that would result in equivalent stress). Production of PCs was then followed over time at these equivalent stress concentrations. The time course of PC production in the tolerant and non-tolerant ecotype was very similar, indicating that PCs had a constitutive and not an adaptive role in Cu tolerance. Molecular studies would need to adopt similar methodologies to establish which genes are differentially regulated in tolerant and non-tolerant ecotypes at equivalent stress (Meharg, 2003). Similar studies identifying oxidative stress enzyme production as a mechanism by which mycorrhizal fungi can deal with redox stress created by toxic metal cations, and thus enhance contaminated site colonisation, have been carried out (Jacob *et al*., 2001; Martino *et al*, 2002; Ott *et al*., 2002).

1.7.2.5 Organic Acid Exudation

Like certain plant hosts, mycorrhizal fungi are able to excrete organic acids into the rhizosphere (Landeweert *et al*., 2001). The benefits of organic acid secretion might be to liberate basic cations from soil minerals (Landeweert *et al*., 2001), to mobilise phosphates from insoluble Fe and Al phosphates, to counteract Al and Fe toxicity by complexing the ions in soil solution, to mobilise trace metal cations by complexing them and to acidify the rhizosphere (Meharg, 2003). Thus with regards to metal tolerance, organic acid exudation may either mobilise metal toxicants in soil, or they may immobilise or detoxify them by complexing them (Meharg, 2003). Organic acids such as citric, malic and oxalic acid are easily utilisable organic substrates which makes the dynamics of these acids in the rhizosphere complex (Jones, 1998). Also of importance in their ability to mobilise/immobilise ions are their affinities for particular metal ions. Such properties are often pH dependant (Meharg, 2003).

Martino *et al*. (2002) analyzed the ability of the ericoid mycorrhizal fungus *Oidiodendron maius,* isolated from metal polluted and unpolluted sites, to mobilize the insoluble salts, zinc oxide and zinc phosphate. It was observed that isolates from non-polluted sites mobilised more Zn from both the salts tested and the enhanced solubilisation was achieved through enhanced excretion of Zn chelating citric and malic acid into experimental culture agar. This was done in comparison to isolates from non-polluted sites with a reduced acid exudation. Acid production was induced when Zn was present but not when Zn was absent. However, it has also been suggested that as in mine spoil and polluted soils many of the toxic metals will be in insoluble forms and therefore not bioavailable. Not enhancing the bioavailability of toxic metal ions may also be a strategic adaptation to grow on contaminated sites (Ahonen-Jonnarth *et al*., 2000). Again, it is apparent that the physiological responses to toxic cations by mycorrhizal fungal isolates varies greatly between isolates and that different metals elicited different excretion responses (Meharg, 2003).

1.7.2.6 Binding to Hyphae

Numerous studies investigating the role of mycorrhizal fungi in enhancing metal resistance of their hosts have concentrated on the extracellular binding of metals to hyphae (Meharg, 2003). Such studies have concentrated on the hypothesis that fungal hyphae act as a filter, removing toxic ions before they reach the plant (Hartley *et al.* 1997). This hypothesis requires: (a) that the metals themselves do not damage the fungal cells; (b) that the metals stay immobilised on the fungal cells and are not in equilibrium with the growth medium; and (c) that the fungal biomass is sufficient to immobilise the metals (Meharg, 2003).

Frey *et al.* (2000) investigating Zn and Cd localisation in *Picea abies* (spruce) root tips associated with *Hebeloma crustuliniforme* (*H. crustuliniforme*), an ectomycorrhizal fungus, found that Cd was predominantly bound extracellularly in the Hartig net, while Zn accumulated mainly in cell walls of mantle hyphae, Hartig net hyphae and in cortical cells. From results obtained, it was proposed that Zn accumulated in high quantities in fungal tissues, protecting the host plant. In a similar study of spruce plants colonised by *H. crustuliniforme*, higher amounts of metals were localised within cross sections of mycorrhizal roots in comparison to non-mycorrhizal roots (Brunner and Frey, 2000). Cd was detected primarily in the Hartig net, Al and Ni in the Hartig net and cortex, with Zn in the Hartig net, cortex and fungal mantle.

It must be noted that the surface area of AMF is a lot smaller compared to ectomycorrhizal fungi and it is therefore likely that metal ion removal from soil to protect their hosts is not as efficient in such associations. However, hyphal binding has been demonstrated to varying extents by AMF. Joiner and Leyval (1997) found that an insensitive *G. mosseae* strain bound three times more Cd to its external surface than the most sensitive strain tested. When ^{109}Cd was used to trace Cd movement through soil into mycorrhizal clover, AMF infection with this insensitive strain enhanced Cd assimilation by the plant roots, but restricted transfer of this Cd to the shoot. These were carefully conducted experiments with only the AMF hyphae having access to the soil compartment containing radioactive Cd, and it was concluded that AM fungal structures in the root were immobilising Cd.

The main pattern that has emerged from such studies is that mycorrhizal fungi show a wide range of physiological responses to metals in the rhizosphere with respect to metal binding or precipitation by hyphae (Meharg, 2003).

1.8 Mycorrhizas in Coal Biotransformation

The ability of certain fungi to effect the biotransformation of coal by involving biosolubilisation and depolymerisation reactions has been the subject of intensive investigation since the late 1970s. Though coalification is accompanied by characteristic changes in the physical properties and chemical structure of plant material, typical structures of the plant material are preserved (Ralph and Catcheside, 1998). The proposition that hard coal might be acted upon by microorganisms was initially made by Hayatsu *et al.* (1979) who linked the structure of hard coal to that of lignin. This led to studies by Fakoussa (1981) who investigating the use of hard coal as a sole C source for microorganisms. Initial results described observations of what were termed brown culture supernatants and subsequent studies indicated that the hard coal particles were partly dissolved (Fakoussa, 1988). Also documented by Cohen and Gabriele (1982) was the dissolution of leonardite (a weathered/highly oxidised lignite) to liquid drops by two fungal species (*Polyporus versicolor* and *Poria monticolar*). Such studies prompted researchers around the world into gaining a deeper insight of the field of coal microbiology and to clarify the microbial mechanism of this effect (Ward, 1985; Scott *et al.* 1986; Strandberg and Lewis, 1987; Wilson *et al.* 1987; Fakoussa 1989).

During an investigation into coal biotransformation activity in South African coal mine dumps, and sampling of microbial populations present, Rose (pers. comm. 2005) reported both the growth of *C.dactylon* directly on coal dump surfaces (Figure 1.1) as well as the degradation of the coal in the rhizosphere of the plants to form a humic soil-like substrate (Figure 1.2).

Figure 1.1. Sporadic growth of *Cynodon dactylon* was observed on the surface of an un-rehabilitated discard coal dump at the Anglo Coal Navigation Site (Witbank, South Africa). The direct growth of a *Cynodon dactylon* plant on discard hard coal is shown in the picture above (Picture: P.Rose, 2005).

Screening studies undertaken by Igbinigie *et al.* (2007) resulted in the isolation of well over 1000 rhizospheric fungal species with some possessing the ability to grow on hard coal as the sole substrate. In these studies the Deuteromycota *Neosartorya fischeri* (*N.fischeri*), Environmental Biotechnology Research Unit (EBRU) Culture Collection Isolate Number 84 (ECCN84), was described for the first time in this environment and was found to be the most active of all the isolates investigated. At the same time, investigation of the *C.dactylon* plants growing on these dumps revealed the presence of mycorrhizal fungi in all the roots of all plants sampled from the dumps investigated (Atkins, pers. comm. 2005). These observations led to the question as to whether interactions between *C.dactylon*, associated mycorrhizal fungi and non-mycorrhizal rhizospheric fungi growing in the rhizosphere of the *C.dactylon* root system may act synergistically to effect the breakdown of coal to form the humic soil-like degradation product observed on these dumps. The question also arose as to whether this system as a whole acted in a manner analogous to “rock-eating” fungal systems previously described (Jongmans *et al.*, 1997).

Figure 1.2. Soil formation due to the action of *Cynodon dactylon* and associated fungi was observed at the Navigation dump site (Witbank, South Africa). The picture reveals the extents of soil formation observed to be occurring within the rhizosphere of *Cynodon dactylon* plants. The system by which this was occurring was investigated in this study (Picture: P.Rose, 2005).

1.9 The Fungcoal Project

Following the initial observations on the Navigation Colliery waste coal dumps described above, the EBRU at Rhodes University commenced an investigation of this system funded by Anglo Coal, a division of the Anglo American Corporation. The study of the microbial ecology of the rhizosphere of *C.dactylon* growing on waste hard coal was undertaken in two parts. The study reported here investigated the mycorrhizal component of the system. The action of non-mycorrhizal rhizospheric fungi was investigated and reported by Igbinigie *et al*. (2007). The underlying objective of this research initiative was to develop possible applications of this system in biotechnology process development based on an understanding of mechanisms involved.

1.10 Hypothesis

AMF present in the rhizosphere of *C.dactylon* growing on hard bituminous coal (HC) waste dumps play a role in interacting with non-mycorrhizal rhizospheric fungi thereby resulting in the degradation of the coal to a humic soil-like substrate and enabling plant growth to establish in this system. This process is comparable to soil formation described in the degradation of minerals by so called "rock-eating" fungi.

1.11 Objectives

This study was undertaken to investigate the proposition that mycorrhizal fungi colonising *C.dactylon* roots growing on coal dumps can interact directly with the coal as well as with other fungi in the rhizosphere to enhance coal degradation, resulting in the production of a humic soil-like substrate over time. The objectives of this study were to:

1. Identify the mycorrhizal fungi present within the *C.dactylon* coal system.

2. Develop an experimental system using pot trials and column reactors in order to simulate the coal dump environment under controlled laboratory conditions.

3. Assess the effects of mycorrhizal and non-mycorrhizal rhizospheric fungi on the breakdown of coal into a humic soil-like substrate.

4. Compare and analyse elemental uptake and distribution within mycorrhizal and non-mycorrhizal components of the *C.dactylon*/coal system that could influence plant and animal toxicity in a potential application of the system.

5. Investigate practical applications of this system in the rehabilitation of waste coal dumps.

CHAPTER 2:

IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGAL ACTIVITY IN THE GROWTH OF *CYNODON DACTYLON* ON A BITUMINOUS HARD COAL SUBSTRATE

2.1 Introduction

Preliminary laboratory studies previously undertaken on the growth of *C.dactylon* directly on waste HC dump material had shown that *C.dactylon* was unable to grow without the addition of a weathered HC (WC) supplement (Atkins, pers. comm. 2005). This work indicated that the supplementation of HC with 10-25% WC as well as the release of humic acids from the WC enabled growth of the grass. It was proposed that the humic acid fraction derived from the WC may provide a nutrient source for the initial establishment of coal-degrading microbial populations in the rhizosphere, which in turn provided conditions that enabled *C.dactylon* to grow in this otherwise hostile environment (Igbinigie *et al*., 2007).

Intensive screening studies of the non-mycorrhizal microbial populations in the rhizosphere of *C.dactylon* had revealed the presence of a number of fungi with the ability to degrade HC (Igbinigie *et al*., 2007). One of these, ECCN84, was utilised in subsequent investigations of coal biosolubilisation and the *C.dactylon* rhizosphere (Igbinigie, 2007). Preliminary studies also reported the presence of extensive root colonisation of *C.dactylon* roots growing directly on HC by AMF.

The study reported in this chapter undertook an investigation of the mycorrhizal component of the *C.dactylon*/coal system in pot trials comparing various forms of treatment. The basic question addressed here was whether or not mycorrhizal fungi exercised any influence on *C.dactylon* growth performance in a HC substrate. Since *C.dactylon* is known to grow well in soil utilised as a cladding material for coal dump rehabilitation, this was used to compare the performance of *C.dactylon* growing directly on HC.

One of the objectives of the project was to determine whether *C.dactylon* growth in HC, with the production of a humic soil-like substrate, could possibly replace the utilisation of high-cost soil cladding in the rehabilitation of waste coal dumps. The ability of this layer to perform in a manner equivalent to soil cladding with the exclusion of oxygen and moisture into discard HC dumps was also subsequently investigated. Given the potential industrial application of these findings, it was thus not only important to understand the role mycorrhizal fungi played in this system but also to establish which combinations of treatments worked best in the establishment of a humic soil-like material layer.

2.2 Methods

2.2.1 Pot Trial Set-Up

Pots (17cm deep and 20cm wide) were surface sterilised in 50% sodium hypochlorite solution for 24 hours and then air-dried. Each pot was 2/3 filled with HC and the top 1/3 of the pot was filled with either a 25% WC supplement or soil. The top layer represented the dump cladding layer in each experiment and the underlying HC below, the discard dump. The HC, WC and soil utilised were obtained from the Anglo Coal Kroomdraai Mine (Witbank, South Africa). The WC supplement utilised in trials consisted of a mixture of sieved (5mm sieve) WC (25%) and HC (75%). The application of this blend was demonstrated in previous studies to yield the best growth of *C.dactylon* (Atkins, pers. comm. 2005).

For treatments requiring inoculation with AMF, 10g of mycorrhizal inoculum, developed by Dr. J. Dames (Mycoroot Pty Ltd., Grahamstown, South Africa), was added to each pot. The mycorrhizal inoculum was developed from *C.dactylon* roots sourced from the Anglo Coal Navigation Mine Site (Witbank, South Africa) where the initial observations of mycorrhizal fungi colonising *C.dactylon* growing on un-rehabilitated coal discard dumps were made. A detailed investigation of the AMF species present in the inoculum is reported in Chapter 3. The inoculum was distributed evenly and lightly covered with cladding material.

For treatments requiring the addition of ECCN84, 50 millilitres (ml) of a spore suspension (50,000 spores/ml) was poured evenly into each pot. Spore suspensions were prepared by washing a fungal mat of ECCN84 grown on Potato Dextrose Agar (Merck, HG00C100.500) with sterile distilled water. *C.dactylon* seed (0.5g) was distributed evenly onto each pot and slightly covered (1cm) with a portion of cladding material. After planting, pots were maintained at a set temperature of 25°C in a constant environment laboratory (Figure 2.1) with a day:night light regime of 16:8 hours provided by banks of Grolux fluorescent tubes linked to an automated timer. Incidental light was approximately 150µmoles/m^2/sec. Each pot was watered daily with 50ml tap water.

The experiment was set up with the following treatments:

1. HC + WC Supplement (+*C.dactylon*, -AMF)
2. HC + WC Supplement (+*C.dactylon*, +AMF)
3. HC + Soil Cladding (+*C.dactylon*, -AMF)
4. HC + Soil Cladding (+*C.dactylon*, +AMF)
5. HC + WC Supplement (+*C.dactylon*, -AMF, +ECCN84)
6. HC + WC Supplement (+*C.dactylon*, +AMF, +ECCN84)
7. HC + Soil Cladding (+*C.dactylon*, -AMF, +ECCN84)
8. HC + Soil Cladding (+*C.dactylon*, +AMF, +ECCN84)

+ : denotes presence
- : denotes absence

For each treatment, 9 replicates (72 pots in total) were planted and pots were harvested and analysed after 22 weeks.

It should be noted that the HC + *C.dactylon* control (*C.dactylon* growing on hard coal without supplementation or treatment) had been consistently shown in previous studies not to yield any plant growth and was therefore excluded in this study (Atkins, pers. comm. 2005).

Figure 2.1. Pot studies were housed in a controlled environment chamber. The various treatments of cladding hard coal with soil or amending it with a weathered coal supplement can be seen.

2.2.2 Analysis

2.2.2.1 Plant Growth and Biomass Yield

Pots were soaked in water to loosen the coal and soil material around the plant roots and each grass plant carefully removed from the pot taking care to keep the roots and shoots intact (Figures 2.2 and 2.3) . The number of plants in each pot was counted and excess coal and soil was washed off the roots with water. Plant roots were then separated from the shoots and the root wet weights measured. The roots and shoots were then dried at 60 degrees Celsius (ºC) for 48 hours. Dry weights of both roots and shoots were measured. Total plant biomass measurements (dry weights) within each pot were then calculated by adding the root weights to the shoot weights. By dividing the total biomass measurements obtained by the number of plants harvested from each pot, an average weight per plant was calculated.

Prior to the drying of root samples, root sub-samples were collected from the roots of each treatment for mycorrhizal colonisation analysis. Predicted dry weights of each root sub-sample taken were then calculated according to percentage moisture content losses observed in whole root sections after drying. The predicted weights of the root sub-samples were then added to the final total biomass of plants.

Figure 2.2. At harvest, whole contents of pots were carefully removed. Shown in the picture is the bituminous hard coal and soil cladding material from a pot that underwent this type of treatment (HC + Soil + *C.dactylon*). Extensive *Cynodon dactylon* root growth into the bituminous coal was observed.

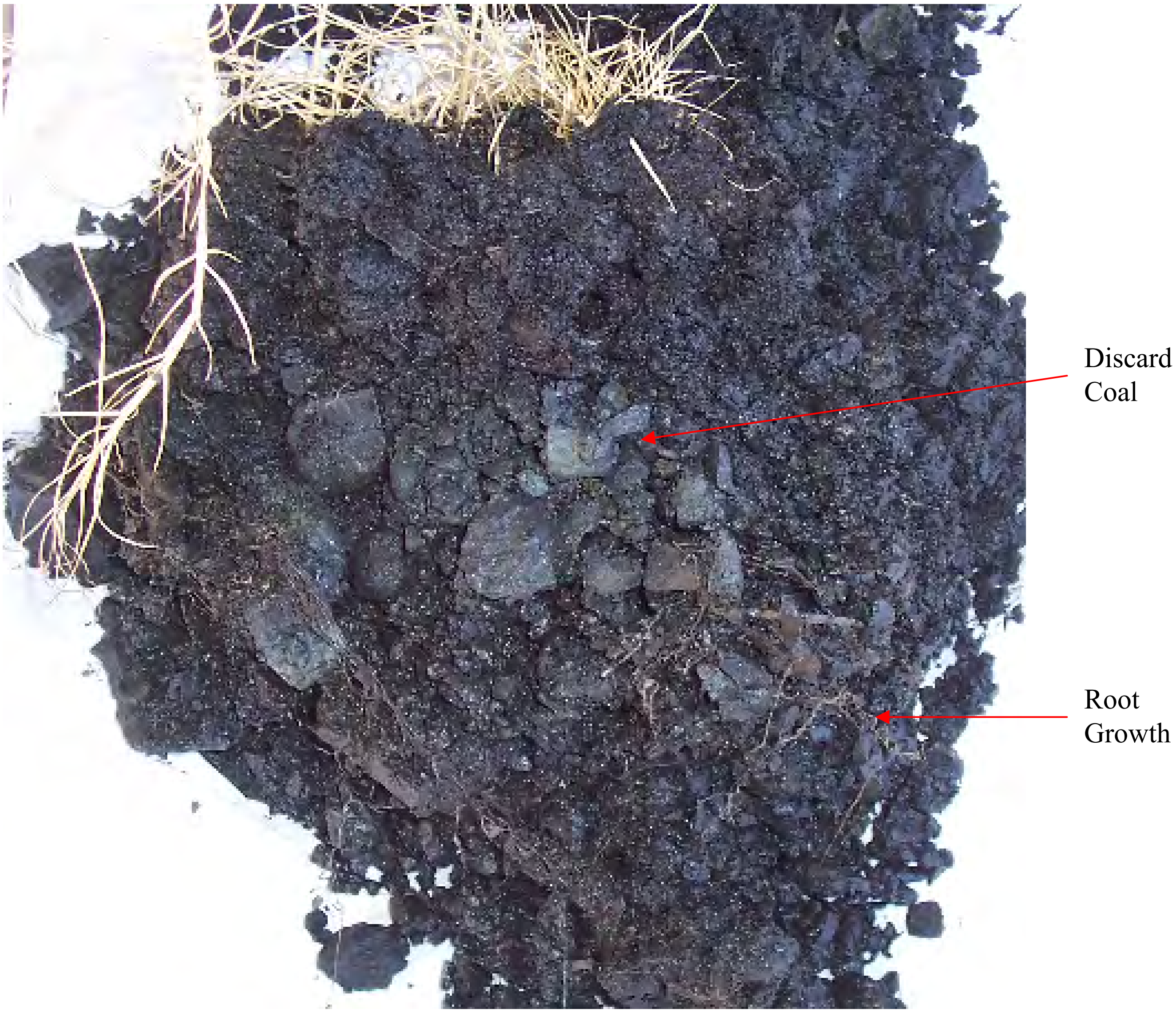

Figure 2.3. At harvest, it was observed that root growth in treatments involving hard coal amended with a weathered coal supplement was reduced in comparison to where soil cladding was utilised. The contents of a treated pot (HC + WC + *C.dactylon*) including root growth within the discard coal can be seen in the picture.

2.2.2.2 Mycorrhizal Colonisation of Cynodon dactylon Roots

Root samples of approximately 0.5g were carefully washed with water and cut into 1-3cm sections. Sections were then covered with 5% potassium hydroxide solution (Merck, 504 44 00 EM) and incubated at 90°C for 45 minutes in a Labcon UOBH301 water bath. The cytoplasm and all coloured material from the plant cells were removed in this process. The potassium hydroxide solution was discarded and the roots rinsed well with distilled water. Samples were then covered with a freshly prepared alkaline hydrogen peroxide solution (Appendix 1) and left to bleach for 60 minutes at room temperature. The bleaching solution was discarded and the root samples rinsed with water. Prior to staining, root samples were acidified in a 0.1M hydrochloric acid solution (Merck, 306 20 LP) overnight to ensure adequate binding of stain to fungal structures. The hydrochloric acid solution was discarded and roots covered with a Lactoglycerol Trypan Blue Stain (Appendix 1) before being incubated for 45 minutes at 90°C. The stain was poured off and root samples allowed to destain in a Lactoglycerol de-stain solution (Appendix 1) for 24 hours prior to microscopic examination with a light microscope (Smith and Dickson, 1997).

Root samples were examined using a Nikon YS100 light microscope and a modified Line Intersect method (McGonigle *et al.,* 1990) from which percentage colonisation, as well as colonisation strategy, were recorded. Lengths of stained roots were laid out onto a slide and covered with a cover slip. One hundred fields of view were examined at 40X magnification and in each view the presence or absence of mycorrhizal structures was recorded. If mycorrhizal structures were observed in the field of view being analysed, a score of 1% was noted. In the absence of any mycorrhizal structures, a 0% score was noted. Observations were recorded using a JVC GC-X3 digital still camera.

2.2.2.3 Glomalin

Replicate 1g coal/soil samples, taken from the interface of the cladding layer and HC in each pot were placed in 50ml centrifuge tubes (Beckman-Coulter) with 8ml 50mM sodium citrate buffer (BDH, BB301284L), pH 7 (Appendix 1). Samples were autoclaved at 121°C for 90 minutes in a Rexall Industries LS-2D autoclave to extract the glomalin. Samples were then immediately centrifuged in a Beckman-Coulter Avanti J-E Centrifuge at 5000rpm for 15 minutes to pellet excess coal/soil particles. The supernatant, containing the protein, was then removed and stored at -20°C prior to analysis (Wright *et al*., 1996).

Glomalin extractions from coal samples contained relatively high humic acid contents. This darkened the colour of the sample solution and affected optical density (OD) readings. The glomalin within each sampled was removed by acetone precipitation. One ml ice cold acetone (Merck, 102 20 40 CC) was added to 200ul of sample and vortexed. This resulted in a clear separation of humic material which sedimented to the bottom of the tube. The top aqueous layer was collected, incubated at -20°C for 10 minutes and finally centrifuged at 13,000rpm for 5 minutes in a J.P. Selecta CE 95 bench-top centrifuge (Bollag *et al.*, 1996). The supernatant was discarded and the pellet air-dried at room temperature before being re-suspended in 20µl of 50mM sodium citrate buffer.

Glomalin concentration was measured utilising the Bradford's Assay (Bradford, 1976). Five µl of sample was added to 250µl Bradford's Reagent (Sigma, B-6916) in a microtitre plate well and allowed to incubate for 5 minutes at room temperature. The OD of the sample was then read at 595nm on a Biotek Instruments Power Wave microtitre plate reader. Standard protein solutions (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6mg/ml) were prepared by diluting the appropriate amount of the Bovine Serum Albumin (Sigma, B4287-16) in distilled water. Five µl of each standard concentration was also incubated with 250µl Bradford's Reagent and the OD of each read at 595nm. A standard curve ($y = 0.5009x$, $R^2 = 0.9743$) was generated from which concentrations of glomalin were determined (Appendix 1).

2.2.3 Statistical Validation of Data

All data is presented as mean values of at least 3 replicate readings with standard errors reported. Significant differences between treatments were tested by one way Analysis of Variance (ANOVA) using Fisher's LSD test. A 95% degree of confidence was utilised where the level of statistical significance was accepted at $p < 0.05$. Statistical analysis was carried out using the STATISTICA V7 (StatSoft Inc., 2005) statistics package (Zar, 1998).

2.3 Results

2.3.1 Plant Growth and Biomass Yield

C.dactylon plants were grown in pot trials under various treatments in order to assess the effect these would have on plant growth and establishment. After 22 weeks, good plant growth was recorded where HC was cladded with soil or amended with a WC supplement (Figures 2.2 and 2.3). However yields were higher in the soil cladded pots than in pots containing the WC supplemented HC. This was more distinct when comparing visual root establishment. Average biomass yields in control treatments of HC + Soil + *C.dactylon* were significantly higher ($p < 0.05$) than biomass yields in the corresponding treatment where a WC supplement was utilised instead of soil (HC + WC + *C.dactylon*). In the latter treatment, the average biomass of plants was 14.9% of plants grown on HC cladded with soil (Figure 2.4 and 2.5).

The highest biomass recorded overall across the series of treatments (0.061g) was from the treatment HC + Soil + *C.dactylon* + AMF (Figure 2.4). Biomass measurements of plants in this treatment increased 27% in comparison to the biomass of control plants (HC + Soil + *C.dactylon*) with yields here being significantly higher than yields obtained in the 3 remaining treatments where soil was utilised as a cladding material. In the corresponding treatment where WC coal supplementation was utilised instead of soil cladding (HC + WC + *C.dactylon* + AMF), average plant biomass yields were only 10.9% of that obtained where soil was utilised as a cladding material.

The lowest average biomass yield obtained where soil was utilised as a cladding material (0.029g) was from the treatment HC + WC + *C.dactylon* + ECCN84. Growth here was 52.3% of that observed in the highest growth yielding treatment of HC + Soil + *C.dactylon* + AMF.

In the final treatment involving HC cladded with soil (HC + Soil + *C.dactylon* + AMF + ECCN84), the effect of AMF inoculation was not as great as the effect of AMF inoculation alone with average biomass yields being lower in comparison (0.044g). Once again, however, biomass yields from this treatment were significantly higher than yields in the corresponding treatment where a WC supplement was utilised (Figure 2.5) which were 27.9% of those in the soil cladded treatment.

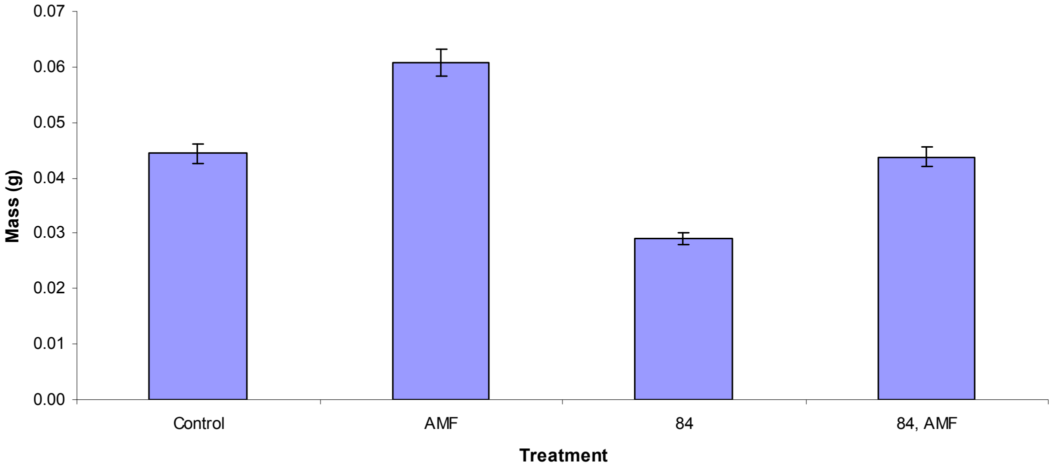

Figure 2.4. Average plant mass yield of *Cynodon dactylon* grown on hard coal cladded with soil after 22 weeks. Treatments compared; Hard Coal + Soil + *Cynodon dactylon*, Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Soil + *Cynodon dactylon* + EBRU Culture Collection Isolate Number 84 and Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84.

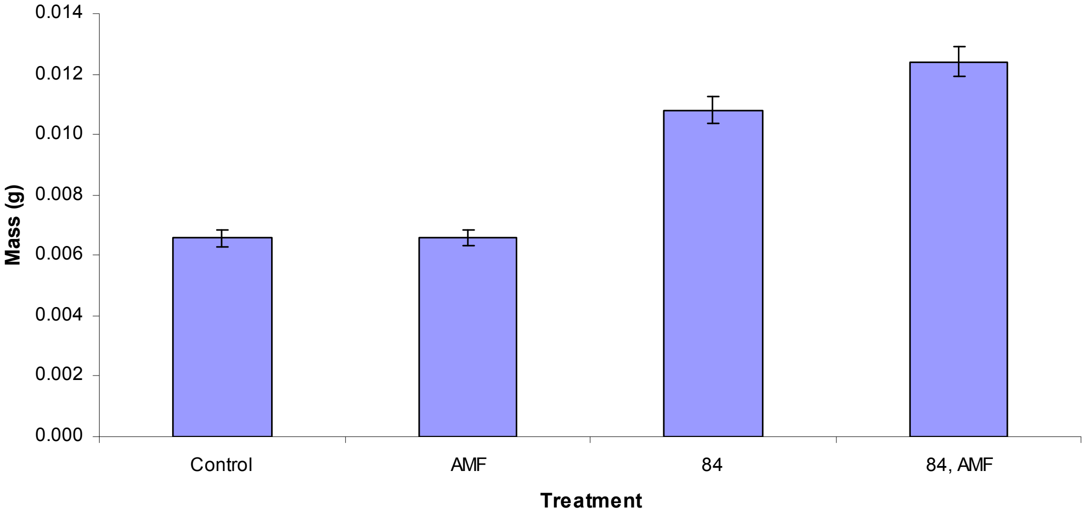

Figure 2.5. Average plant mass yield of *Cynodon dactylon* grown on hard coal supplemented with weathered coal after 22 weeks. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + EBRU Culture Collection Isolate Number 84 and Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84.

While the addition of AMF significantly increased *C.dactylon* growth performance in the soil cladding pots, the addition of AMF to the WC supplemented system did not significantly increase plant biomass yields in comparison to the control. Yields from both these treatments were similar (Figure 2.5). The addition of

ECCN84 to the WC supplemented system (HC + WC + *C.dactylon* + ECCN84) was observed to increase plant biomass yields by 39.3% in comparison to control treatments. This however was not found to be a significant increase. The only statistically significant increase in plant biomass yield in comparison to the control for the WC supplementation studies was from the treatment HC + WC + *C.dactylon* + AMF + ECCN84 with yields here being the highest across the series of treatments (0.012g). A 52.9% increase in growth was measured in comparison to the control. The 12.9% increase in growth recorded from this treatment in comparison to the treatment involving inoculation solely with ECCN84 (HC + WC + *C.dactylon* + ECCN84) was found not to be a significant increase (Figure 2.5).

2.3.2 Mycorrhizal Colonisation of *Cynodon dactylon* Roots

The effect of various pot trial treatments on root mycorrhizal colonisation was analysed microscopically with colonisation percentages related to degrees of mycorrhizal establishment. Levels of root mycorrhizal colonisation observed for trials involving *C.dactylon* growth on HC cladded with soil as well as HC amended with a WC supplement are presented in Table 2.1. Levels of mycorrhizal colonisation, where recorded, were higher in the roots of *C.dactylon* plants growing on HC supplemented with WC in comparison to in the roots of plants growing on HC cladded with soil.

In trials where *C.dactylon* was grown on HC cladded with soil, no mycorrhizal colonisation was recorded from the un-inoculated control treatment (HC + Soil + *C.dactylon*). The highest root colonisation percentage recorded was from the treatment involving inoculation with both AMF and ECCN84 (8.33%). This was significantly higher ($p < 0.05$) than colonisation percentages recorded in control treatments, as were colonisation percentages in the remaining 2 treatments (HC + Soil + *C.dactylon* + AMF, HC + Soil + *C.dactylon* + ECCN84). Mycorrhizal colonisation was observed in the treatment HC + Soil + *C.dactylon* + ECCN84, a treatment to which mycorrhizal inoculum was not added.

In trials where *C.dactylon* was grown on HC supplemented with WC, the mycorrhizal colonisation was higher with 19.33% recorded in the treatment involving inoculation of the system solely with AMF (HC + WC + *C.dactylon* + AMF). This was significantly higher ($p < 0.05$) than colonisation percentages recorded in the control treatment (0.33%). Also significantly higher in comparison to the control was the colonisation percentage in the treatment HC + WC + *C.dactylon* + AMF + ECCN84 (18.33%). The difference in colonisation percentages between the treatments of HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84 was not significantly different. As in trials where soil was utilised as a cladding material, some limited colonisation was observed in treatments not directly inoculated with AMF (HC + WC + *C.dactylon*, HC + WC + *C.dactylon* + ECCN84).

Barring the control treatments, mycorrhizal colonisation percentages across the series of treatments where *C.dactylon* plants were grown on HC amended with a WC supplement were significantly higher than the corresponding treatments where *C.dactylon* was grown on HC cladded with soil.

Table 2. 1. Percentage root colonisation by arbuscular mycorrhizal fungi of *Cynodon dactylon* plants grown in hard coal either cladded with soil or amended with a WC supplement. Trials involved analysis of un-inoculated control plants, plants inoculated with arbuscular mycorrhizal fungi, plants inoculated the EBRU Culture Collection Isolate Number 84 as well as plants inoculated with both arbuscular mycorrhizal fungi AMF and the EBRU Culture Collection Isolate Number 84.

Treatment	Average AM fungal Root Colonisation Percentage
HC + Soil + *C.dactylon*	0%
HC + Soil + *C.dactylon* + AMF	4.67%
HC + Soil + *C.dactylon* + ECCN84	3.33%
HC + Soil + *C.dactylon* + AMF + ECCN84	8.33%
HC + WC + *C.dactylon*	0.33%
HC + WC + *C.dactylon* + AMF	19.33%
HC + WC + *C.dactylon* + ECCN84	0.67%
HC + WC + *C.dactylon* + AMF + ECCN84	18.33%

Paris-type mycorrhizal colonisation (Figure 2.6) was predominantly observed in *C.dactylon* roots of plants grown on HC amended with a WC supplement. Hyphal coiling was readily encountered in root cells with arbuscule formation being limited. Arbuscules were only observed in treatments involving *C.dactylon* growth on HC cladded with soil.

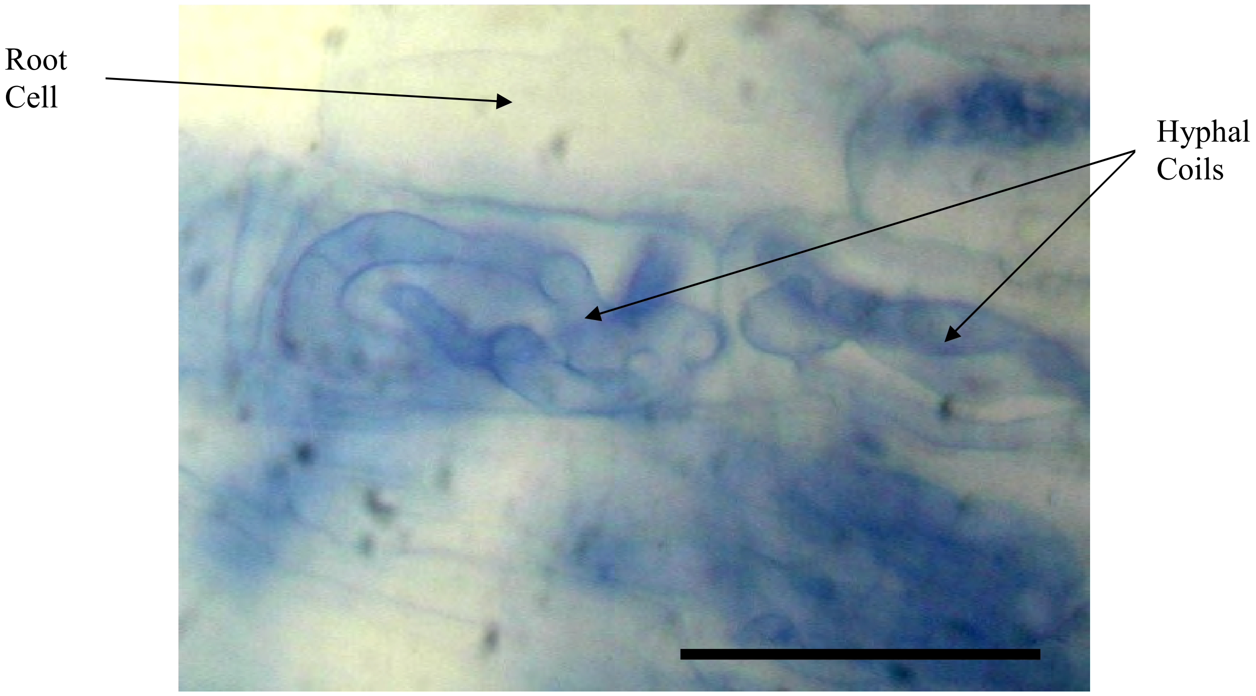

Figure 2.6. Micrograph showing hyphal coiling by mycorrhizal fungi in root cells of *Cynodon dactylon* grown in hard coal amended with a weathered coal supplement and inoculated with arbuscular mycorrhizal fungi. Scale bar = 25µm.

2.3.3 Glomalin

Mycorrhizal fungal establishment within the rhizosphere of the various pot trial treatments was analysed using the detection and quantification of glomalin, the glycoprotein produced predominantly by the extra-radical hyphae of mycorrhizal fungi (Wright and Upadhyaya, 1998).

Where *C.dactylon* was grown on HC cladded with soil (Figure 2.7), the highest glomalin concentrations were recorded from the treatment inoculated with AMF (0.159mg/ml) alone. Glomalin concentrations in this treatment were significantly higher ($p < 0.05$) than in the HC + Soil + *C.dactylon* + ECCN84 treatment (0.037mg/ml), with differences between these two treatments being the only significant differences found. A 43.8% reduction in rhizospheric glomalin concentration was observed where ECCN84 was added to the HC alongside AMF (HC + Soil + *C.dactylon* + AMF + ECCN84) in comparison to the treatment where AMF was added alone (HC + Soil + *C.dactylon* + AMF). The lowest average glomalin concentrations recorded across the series of treatments was from the treatment HC + Soil + *C.dactylon* + ECCN84. The average concentration here was lower than concentrations in the control treatment.

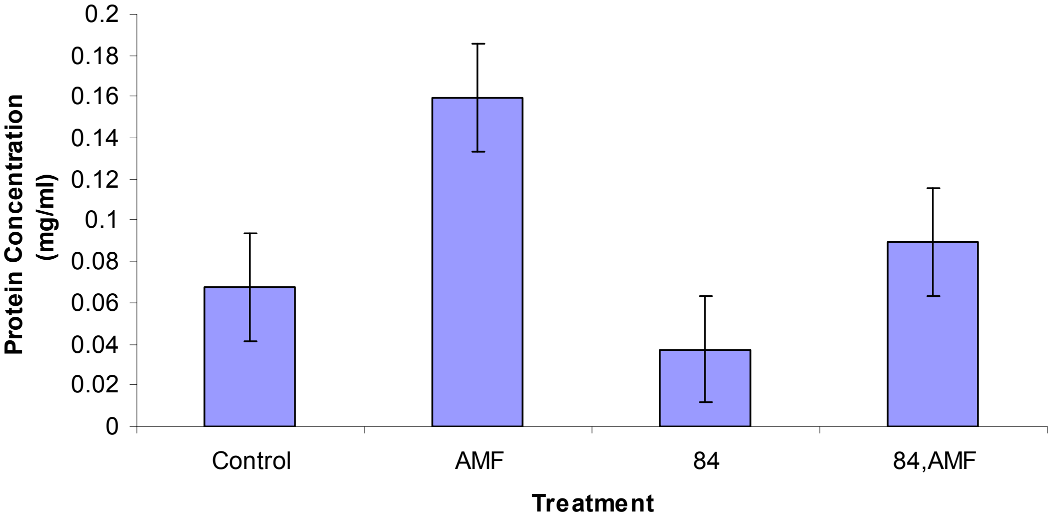

Figure 2.7. Glomalin concentrations in 1g coal samples harvested from the rhizosphere of *Cynodon dactylon* plants grown on hard coal cladded with soil after 22 weeks. Treatments compared; Hard Coal + Soil + *Cynodon dactylon*, Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Soil + *Cynodon dactylon* + EBRU Culture Collection Number 84 and Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84.

Analysis of the rhizosphere of *C.dactylon* plants grown on HC amended with a WC supplement showed some variation in glomalin concentrations across the series of treatments. However these differences were not significant (Figure 2.8). Across these treatments, the average glomalin concentrations was highest in the control treatment HC + WC + *C.dactylon* (0.149mg/ml) followed by the treatment involving inoculation of the rhizosphere with AMF (0.132mg/ml). Where ECCN84 was added to the rhizosphere alongside AMF, an average 23.7% reduction in rhizospheric glomalin concentration was recorded. As with trials involving HC cladded with soil, the treatment of HC + WC + *C.dactylon* + ECCN84 produced the lowest average amounts of glomalin across all treatments.

When comparing glomalin concentrations across both sets of treatments, a 54% reduction in the control treatment was observed where HC was supplemented with WC (HC + WC + *C.dactylon*) in comparison to the corresponding treatment where soil was used as a cladding material. However, given the large standard error, this reduction was statistically significant ($p < 0.05$). The only significant reduction in glomalin concentration was recorded where ECCN84 was added to HC supplemented with WC in comparison to HC cladded with soil. In comparison to corresponding treatments where soil was utilised as a cladding material, glomalin concentrations in the WC supplemented treatments involving inoculation with AMF, HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84, were lower yet comparable.

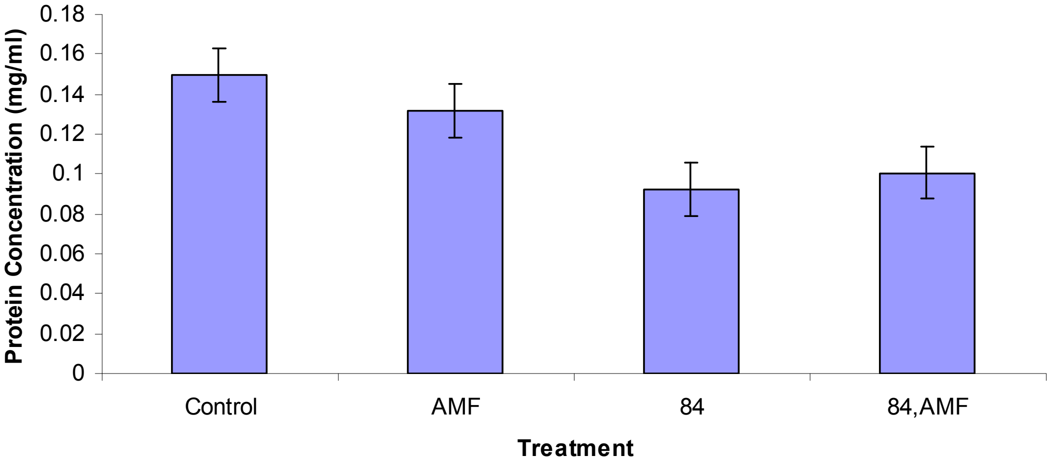

Figure 2.8. Glomalin concentrations in 1g coal samples harvested from the rhizosphere of *Cynodon dactylon* grown on HC supplemented with WC after 22 weeks. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + EBRU Culture Collection Number 84 and Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84.

2.4 Discussion

2.4.1 Plant Growth and Biomass Yield

Arbuscular mycorrhizal associations have been shown to be beneficial to plants in a variety of ways. The principle means by which AMF benefit their host is by increasing the uptake of P primarily due to the ability of AMF mycelia to grow beyond the nutrient depletion zone that will often develop around the plant root (Sanders and Tinker, 1971; Koide, 1991; George *et al*., 1995). The enhanced uptake of P due to the mycorrhizal association can be associated with increased growth (Vostake, 1995; Ibibijen *et al*., 1996; Koide *et al*., 2000).

C.dactylon biomass yield results obtained from pot trials involving the cladding of HC with soil in various treatment combinations showed that plants inoculated with AMF produced the highest biomass yields (Figure 2.4). The results suggest that the mycorrhizal association played a role in producing the enhanced plant effect observed.

Where soil was used and *C.dactylon* inoculated with a combination of both AMF and ECCN84, the effect of AMF on plant growth appeared to have been reduced with biomass yields similar to those of the un-inoculated plants (Figure 2.4). With enhanced nutrient acquisition being one of the key methods by which AMF enhance plant growth, the results of this study could indicate a possible competition for nutrients between AMF and ECCN84. The utilisation of rhizospheric nutrients by ECCN84 may have resulted in a reduction in plant nutrient acquisition and thus plant growth. The fact that the lowest total biomass measurements were recorded from the treatment involving inoculation solely with ECCN84 enhances this possibility. Inoculation with AMF most likely partially counteracted this nutrient depletion through enhanced nutrient uptake thus increasing plant biomass yields in comparison to yields in the treatment HC + Soil + *C.dactylon* + AMF + ECCN84. It is also possible that rhizospheric fungi, such as ECCN84, may partially inhibit AMF activity. This in turn may require a higher colonisation rate for the system to function, which was observed (Table 2.1), especially in treatments involving HC supplementation with WC, conditions similar to those encountered at the Navigation Mine site from which ECCN84 was isolated.

In pot trials where *C.dactylon* was grown on HC amended with WC, significant reductions in plant biomass yields were observed across all treatments in comparison to the corresponding biomass yields of plants grown on HC cladded with soil (Figure 2.5). Rhizospheric conditions, which may be anticipated to be harsher in HC substrate/WC than in soil, probably influenced plant growth in this environment. Nutrient and elemental compositions of the WC and soil utilised in these trials, as well as a possible influence on plant growth are discussed in Chapter 5.

In pot trials involving growth of *C.dactylon* on HC amended with a WC supplement, results suggest that the presence of ECCN84 within the rhizosphere enhanced plant biomass yield. This yield was further enhanced by the presence of both AMF and ECCN84 with yields from the treatment HC + WC + *C.dactylon* + AMF + ECCN84 being highest (Figure 2.5). This result suggests that the presence of coal-degrading microorganisms, such as ECCN84, in the *C.dactylon* rhizosphere may play an important role, together with AMF, in mobilising nutrients from the coal substrate.

2.4.2 Mycorrhizal Colonisation of *Cynodon dactylon* Roots

Examination of *C.dactylon* roots sampled from pot trials involving the WC supplement showed that mycorrhizal colonisation in the roots of plants inoculated solely with AMF and plants inoculated with a combination of both AMF and ECCN84 had substantially higher mycorrhizal colonisation than control plants and plants inoculated solely with ECCN84 (Table 2.1). The low, yet present, colonisation observed in treatments not inoculated with AMF could be explained as a result of background mycorrhizal fungal populations within the substrates utilised in trials. AMF are widely distributed in nature and present in a wide variety of ecological niches (Smith and Read, 1997; Chaurasia *et al*., 2005).

Analysis of roots from treatments where *C.dactylon* was grown on HC cladded with soil surprisingly revealed lower colonisation in comparison to the roots of plants grown on HC amended with a WC supplement (Table 2.1). It has been reported that the infectivity, propagation rates and nutrient transfer to the plant host by particular AMF species and strains can be highly influenced by soil conditions, with the strategy of adaptation being a factor to variations in mycorrhizal activity observed (Del Val *et al*., 1999; Orteg Larocea, 1999; Mathimaran *et al*., 2006). The harsher conditions prevalent in the HC substrate compared to soil may have resulted in an increased colonisation of *C.dactylon* to enable growth in this system.

Rhizospheric conditions may have also played a role in the colonisation patterns observed within the roots from the various treatments. Hyphal coiling was observed (Figure 2.6) within roots across all treatments. Limited arbuscule formation was observed the roots of plants from treatments involving cladding of HC with soil. Arbuscules formed by mycorrhizal fungi within plant cortical cells are short lived with the life span thought to be a few days. After this, they collapse and degrade, enabling another arbuscule to develop within the undamaged cell (Alexander *et a*l., 1988; Alexander *et al*, 1989). In contrast, the colonisation strategy where hyphal coils are formed is regarded as being longer-lasting and is associated with slower-growing plants (Smith and Read, 1997; Yamato, 2004). Colonisation strategies within plant roots can be highly influenced by rhizospheric conditions (Harrison, 1999) and it would appear that the environment provided by the cladding material utilised in this study influenced the colonisation strategies observed. In the absence of soil, the AMF present in the rhizosphere most likely formed hyphal coils rather than arbuscules and this colonisation pattern may reflective of the harsher conditions encountered in the HC/WC system.

2.4.3 Glomalin

During an attempt to analyse the efficiency of an immunological probe for hyphae of AMF, Wright *et al.* (1996) discovered that a protein is produced by these organisms in relatively high abundance on actively growing hyphae. The protein, produced predominantly on extra-radical hyphae, was subsequently called “glomalin” from Glomales, the taxonomic order in which AMF were classified at the time (Wright and Upadhyaya, 1996). Glomalin is insoluble in water and the extraction of this protein was observed to require harsh conditions, namely sodium citrate at an alkali pH and a temperature of 121°C (Wright *et al.,* 1996). Such extreme conditions of extraction indicate the stability of this molecule (Wright and Upadhyaya, 1996). Glomalin is a glycoprotein with N-linked oligosaccharides and has some characteristics related to hydrophobins, small proteins produced by fungi (Wessels, 1996).

Estimates of extra-radical hyphal lengths range widely. Hyphal lengths of 111metres (m) per cm^3 of soil have been reported in prairie communities from which a hyphal dry weight of less than 0.5mg/g of soil was calculated (Miller and Jastrow, 1990). In other studies, Olsson *et al.* (1999) analysed soil from a linseed field and using phospholipid fatty acid analysis, measured hyphal dry weights of 0.03-0.035mg/g present within the soil. They also found that the AMF dry weight was the highest soil microbial biomass component of the linseed field.

Initial studies by Wright and Upadhyaya (1996) on the extraction and quantification of glomalin utilising the Bradford’s assay revealed glomalin concentrations in soil ranging from around 0.5mg/g to 12mg/g with the majority of soils having glomalin concentrations of between 2mg/g and 4mg/g.

Glomalin analysis of substrates in the pot trials reported here revealed relatively low levels of glomalin. With both sets of treatments, glomalin levels of between 0.16 and 0.04mg/ml were measured. The highest glomalin concentration was recorded where soil cladding was utilised, as expected in the treatment involving direct mycorrhizal inoculation (HC + Soil + *C.dactylon* + AMF), with concentrations reducing in both sets of treatments where ECCN84 was added to treatments and lower where a WC supplement was utilised. These results suggest that extraradical hyphal growth into the surrounding substrate may have been reduced where ECCN84 was added to the system. Where a WC supplement is utilised, the operation of a dual population system where coal-degraders contribute to the plants nutrition may reduce the need for as an extensive a network of extraradical AMF hyphae as is found in soil alone. In contrast, the increased colonisation observed (Table 2.1) may indicate a strategy enhanced focus on intraradical activity required to sustain the *C.dactylon*/coal system.

The Bradford's assay utilised to quantify glomalin concentrations in extracted samples is based on the assumption that the vast majority of proteins are denatured and degraded in the extraction process that involves extremely high temperatures. Studies have demonstrated, though, that this is not entirely the case with non-glomalin proteins, such as BSA, being recovered using Bradford's assays in spiked samples that have undergone glomalin extraction treatment (Rosier *et al.*, 2006). It is clear that the Bradford's assay does not discriminate between glomalin and proteins. A monoclonal antibody (MAb32B11) has been developed for the specific detection of the glomalin protein using an Enzyme-Linked Imunnosorbent Assay (ELISA). However, due to unavailability, the antibody was not used in this study.

2.5 Conclusions

- The inoculation of AMF enhanced *C.dactylon* establishment on HC cladded with soil or amended with a WC supplement.

- The addition of a non-mycorrhizal rhizospheric fungal isolate from the *C.dactylon*/coal system, ECCN84, resulted in increased biomass production by the grass in its growth on HC amended with a WC supplement.

- An apparent inverse relationship between AMF colonisation of *C.dactylon* and glomalin production in the presence of ECCN84 suggests AMF extraradical hyphal growth may be influenced by a dual fungal population. The coal degraders may contribute nutrients from coal breakdown and the AMF actively transport organic C from the plant into the rhizosphere and nutrients back to the plant. These functions are more enhanced in the WC supplemented system than the soil cladding system and resulted in reduced extraradical establishment by the associated AMF.

- The suggestion that successful establishment of *C.dactylon* growth in a hostile HC environment is enabled by a dual fungal system requires further detailed investigation.

CHAPTER 3:

CHARACTERISATION OF FUNGAL POPULATIONS ASSOCIATED WITH *CYNODON DACTYLON* GROWING ON BITUMINOUS HARD COAL

3.1 Introduction

Studies reported in Chapter 2 had demonstrated the presence of a mycorrhizal fungal population in the *C.dactylon*/HC system as had been described to occur on the Navigation Mine site waste HC dumps. It was shown that the inoculation of the system with AMF positively influenced plant growth with the mycorrhizal colonisation of *C.dactylon* demonstrated. The active role of a coal-degrading non-mycorrhizal rhizospheric fungal population within the system was also suggested. ECCN84 was an isolate identified as possessing coal-degrading abilities (Igbinigie, 2007) and its addition to pot trials coupled with enhanced plant growth had further indicated a possible role for this group.

While the experimental results described above were indicative, it would be necessary to characterise the microbial populations involved in order to strengthen the conclusions drawn. In this regard, it would be useful in attributing a causal role for these populations by following the requirements of Koch's postulates as far as possible (Stanier *et al.*, 1970). Although initially established to guide the investigation of disease-causing microbes, the postulates may be applied to an environmental application as follows:

1. The microorganism must be found where a particular effect is recorded;
2. The microorganism must be isolated from this environment;
3. The microorganism should cause the same effect when introduced back into the same environment;
4. The microorganism must be re-isolated from the environment and be identified as the original agent causing the observed effect.

The objective of this experiment was to characterise the mycorrhizal and non-mycorrhizal fungal species present within the *C.dactylon*/HC system established in the pot trails reported in Chapter 2. Elements of Koch's postulates would be followed as a guide to experimental design and also strengthen the possible causal relationship proposed in linking these populations to changes in the *C.dactylon*/coal dump system.

3.2 Methods

3.2.1 Most Probable Propagule Number

Determination of the number of propagules in the mycorrhizal inoculum followed the Most Probable Number (MPN) method of Smith and Dickson (1997). A series of ten-fold dilutions (1:10, 1:100, 1:1000 and 1:10000) of samples were prepared by diluting the mycorrhizal inoculum used with pasteurised soil by volume. Torpedo tubes (4cm x 23cm) were washed in 50% sodium hypochlorite solution and allowed to dry prior to each dilution mixture being packed into each of 5 torpedo tubes. Five Sudan grass (*Sorghum vulgare var. sudanense*) seeds were placed in each torpedo tube and lightly covered with soil. Sudan grass is the standard grass species used in MPN calculations (Smith and Dickson, 1997). Seeds were allowed to germinate and grow for 6 weeks prior to examination. Seedlings were watered once daily with UV sterilised water and kept at a temperature range of 18 to 25°C.

The presence of infective mycorrhizal propagules was detected by clearing and staining the roots prior to microscopic examination as previously described (Section 2.2.2.2). Determination of the presence or absence of mycorrhizal colonisation can be used to calculate the number of propagules present in the inoculum. The MPN is calculated from the numbers of root systems in successive dilutions with and without mycorrhizal fungi using equations based on probability theory (Smith and Dickson, 1997).

3.2.2. Morphological Characterisation of the Mycorrhizal Inoculum

Mycorrhizal isolates present in the inoculum developed from the root zone of *C.dactylon* growing on the waste HC dump (Navigation) by Dr. J. Dames (Mycoroot Pty Ltd.) were initially identified morphologically based on spore characteristics.

3.2.2.1 Spore Extraction

Distilled water (200ml) was added to 100g of mycorrhizal inoculum and the suspension agitated for 5 minutes before being allowed to settle for 15 seconds. The supernatant was decanted through a nest of soil sieves of mesh size 425µm, 250µm, 125µm and 45µm, respectively. This was repeated until water resulting from agitation was clear. The nest of sieves were then gently washed with water before the debris from each of the 3 smallest sieves was washed into separate 50ml centrifuge tubes and filled with distilled water to the 50ml mark.

Tubes were centrifuged at 1900rpm for 5 minutes and the supernatant carefully discarded. The pellet was re-suspended in 60% sucrose solution and centrifuged for a further 5 minutes at 1900rpm. The supernatants (containing spores) from each tube were then separately decanted into a fresh 45µm sieve and rinsed with water to remove any sucrose solution. A 9cm Whatman #1 filter paper disk with a 1cm x 1cm printed grid was placed in a Buchner funnel to which a suction pipe was attached to the bottom. Debris from each tube were separately washed from the 45µm sieve onto the filter paper with distilled water and briefly rinsed. The filter paper was finally transferred to the lid of a clean Petri dish and the dish closed (Smith and Dickson, 1997).

3.2.2.2 Spore Selection and Identification

Extracted spores, with differing morphological characteristics, were selected using a Leica S4E dissecting microscope. On selection, non-parasitised spores were picked and mounted on microscope slides in polyvinyl–lactic acid–glycerine (PVLG) (Koske & Tessier, 1983) or in a 1:1 mixture (v:v) of PVLG with Melzer's reagent

(Brundrett *et al.*, 1994) for further examination (Appendix 2). PVLG is a common microscope mounting medium and Melzers reagent is a stain used to examine fungal cells. Parasitised spores were identified based on spore discoloration, excessive opaqueness and signs of bacterial or fungal growth on the spore surface. Selected spores were examined at 100X magnification using Nikon YS100 light microscope. Spore (species) identification was based on current species descriptions available online at the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM). Morphological differences such as size, shape, colour, hyphal attachment and spore wall characteristics were used to differentiate between spore species. Observations were recorded using a JVC GC-X3 digital camera.

3.2.3 Molecular Characterisation of the Mycorrhizal Inoculum

The mycorrhizal fungal population present within the inoculum utilised in pot trials was investigated using molecular techniques.

3.2.3.1 Genomic Extraction

Total Deoxyribonucleic Acid (DNA) genomic extractions from the mycorrhizal inoculum were undertaken following the methods of Bond *et al*. (2000). A 1g sample of inoculum was diluted in 5ml of Phosphate Buffered Saline (Appendix 2) in a 15ml centrifuge tube (Eppendorf). The sample was vortexed vigorously using a Scientific Industries Vortex Genie-2 before 500µl was transferred to a sterile 1.5ml micro-centrifuge tube (Eppendorf). The sample was centrifuged in an Eppendorf 5415 D bench-top centrifuge at 13,000rpm for 2 minutes and the supernatant discarded. The resulting pellet was re-suspended in 250µl 50% glycerol (Merck, 267 65 20 LC) and 250µl Buffer A (Appendix 2) before the sample was re-centrifuged at 13,000rpm for 2 minutes. The supernatant was once again discarded and the pellet re-suspended in 250µl Buffer A. Cell lysis was initiated by the addition of 15µl of 50mg/ml Lysozyme (Merck, K3344781 502) to the sample and the sample incubated at 37°C for 1 hour in a Labcon UOBH301 water bath. Fifteen µl 50µg/ml Proteinase K (Roche, 1 000 144) was added to the sample and the sample incubated at 50°C for 1 hour to allow for cell protein digestion. Cellular fracture was induced using 4 freeze-thaw cycles of freezing in liquid N prior to thawing at 90°C in a Techne Dri-Block DB-2A heating block. Further mechanical cell wall disruption was undertaken with the addition of 5 sterile glass beads (1mm in diameter) to each sample directly in the microcentrifuge tube, and the tube vortexed for 1 minute. The liquid portion of the sample (minus glass beads) was transferred to a new sterile 1.5ml microcentrifuge tube. To denature and remove proteins from samples, 250µl 10% sodium dodecyl sulphate (BDH, 301754L), 250µl phenol solution (Sigma-Aldrich, P-4557) and 250µl 24:1 chloroform (Merck, 159 50 40 LC):isoamyl alcohol (BDH, 100383L) was added to the sample (Appendix 2). The sample was then vortexed and centrifuged at 13,000rpm for 2 minutes. The upper aqueous layer containing the nucleic acids was removed, placed in a fresh 1.5ml microcentrifuge tube and 500µl chloroform:isoamyl alcohol (24:1) added to the sample to purify the DNA. The sample was vortexed and centrifuged at 13,000rpm for 2 minutes. The upper aqueous layer containing DNA was once again removed and placed in a fresh 1.5ml microcentrifuge tube. To precipitate the DNA in samples, 50µl 3M sodium acetate (Analytical Reagent, RPCK2876) was added to the sample and the tube filled with 96% ethanol before incubation at -20°C overnight (Appendix 2).

Samples were centrifuged in an Eppendorf 5810R Bench-Top Centrifuge at 13,000rpm for 25 minutes at 4°C. The supernatant was poured off and the resulting pellets air-dried before being re-suspended in 50µl sterile distilled water and stored at -20°C.

3.2.3.2 Visualisation of Genomic Extracts

Nucleic acid extracts were visualised using agarose gel electrophoresis. A 1% Agarose gel was prepared consisting of 1g molecular grade agarose (Hispanagar, H0901031) in 100ml 0.5X TAE buffer (Appendix 2) diluted from 50X TAE buffer stock (BioRad, 161-0773). The mixture was boiled until the agarose was completely dissolved and allowed to cool to approximately 40°C. On cooling 100µl 500mg/ml Ethidium Bromide (Appendix 2) was added to the agarose. The agarose was poured into a gel mould and a comb placed into the gel before it was allowed to set. Once set, the gel was placed in a gel tank filled with 0.5X TAE Buffer.

When loading the gel, 10µl of extracted genomic DNA was mixed with 2µl DNA loading buffer and loaded into a well in the gel. Simultaneously, 10µl of λ*Pst1* molecular marker (Appendix 2) was loaded into the gel to determine the molecular weights of the bands obtained on the gel. The gel tank was connected to a BioRad Power Pac 300 and the gel electrophoresed for 30 minutes at 100 volts (V). Gels were visualised using a UVP BioDoc-It Transilluminator System.

3.2.3.3 Polymerase Chain Reaction Amplification of Mycorrhizal Fungal Ribosomal DNA.

The NS31 (Simon *et al.*, 1992) and AM1 (Helgason *et al*, 1998) primer set were used for polymerase chain reaction (PCR) amplification of ribosomal DNA (rDNA) present within DNA extracts. This primer set amplifies a portion of the AM fungal small sub-unit ribosomal DNA (Douhan *et al.*, 2005).

PCR amplification using extracted genomic DNA was carried out using a *Taq* DNA Polymerase kit (Promega, M2861). 25µl PCR reactions were set up containing 2µl genomic DNA, 2µl 10mM AM1 Primer, 2µl 10mM NS31, 1µl 10mM DNTPs (Promega), 1µl 50mg/ml bovine serum albumin (Sigma, B4287-16), 2µl 25% dimethyl sulphoxide (Merck, AA802912.500), 2.5µl Magnesium-free buffer (Promega, M190G), 1.5µl 25mM $MgCl_2$ (Promega, A351H), 0.2µl *Taq* polymerase (Promega, M186E) and 10.8µl sterile distilled water.

PCR reactions were carried out in an Applied Biosystems GeneAmp 9700 PCR Thermocycler. An initial denaturation step at 94°C for 2 minutes was followed by 30 cycles of denaturation at 94°C for 2 minutes, 1 minute annealing at 48°C and 8.5 minutes elongation at 72°C. A final elongation step at 72°C for 8.5minutes completed the reaction. PCR products were stored at -20°C.

A 1% agarose gel was prepared and PCR products were electrophoresed at 120V for 45 minutes using a Bio-Rad Power Pac 300 prior to visualisation on a UVP BioDoc-It Transilluminator System.

3.2.3.4 Ligation of PCR Products

Prior to sequencing, individual PCR products derived from genomic extractions on the various treatments were ligated into the pGEM-T Easy vector supplied in the

Promega pGEM-T Easy Vector System Kit (A1360). Ligation reactions (2µl) were set up in sterile 100µl tubes containing 0.6µl PCR product, 1µl ligation buffer, 0.2µl ligase and 0.2µl pGEM-T vector. Samples were incubated at 4°C overnight.

3.2.3.5 Preparation of Competent Escherichia coli JM109 Cells

A test tube containing 5ml sterilised Luria Bertani Broth (Merck, C150) was inoculated with an *Escherichia coli* (*E.coli*) JM109 colony and grown overnight at 37°C with shaking at ~150rpm in a Labcon shaking incubator. 0.3ml, 0.7ml, 1ml and 1.5ml of the broth respectively was added to each of 4 different 250ml conical flasks containing sterilised 100ml Luria broth. Each flask was incubated at 37°C with shaking at ~150rpm for 2 hours. Cultures were allowed to grow until the OD_{600} of the 1.5ml inoculated flask was recorded to be between 0.6 and 0.8. Flasks were then incubated on ice for 10 minutes.

From each culture, 50ml of broth was transferred into a sterile 50ml centrifuge tube (Beckman) and cultures centrifuged at 5000rpm for 10 minutes in a Beckman-Coulter Avanti J-E Centrifuge using a JA-14 rotor. The supernatant was discarded and the pellet re-suspended in 50ml RF1 solution (Appendix 2) before the tubes were incubated on ice for 20 minutes. Tubes were once again centrifuged at 5000rpm for 10 minutes at 4°C and each pellet re-suspended in 4ml RF2 solution (Appendix 2). Re-suspended solutions were pooled and 150µl volumes of prepared cells aliquoted into sterile microcentrifuge tubes and stored at -80°C (Sambrook *et al.*, 1989).

3.2.3.6 Transformation of Competent Cells with Ligated Clones

Ligation reactions (2µl) previously incubated at 4°C overnight were added to 50µl thawed competent *E.coli* cells in a sterile 1.5ml microcentrifuge tube and incubated on ice for 20 minutes. To allow for plasmid uptake, samples were heat-shocked at 42°C in a Labcon UOBH301 water bath for 45 seconds before being incubated on ice for a further 5 minutes. Nine hundred µl SOC media (Sigma, S-1797) was added to the transformed cells and the cells incubated at 37°C with shaking at 150rpm for 1 hour in a Labcon shaking incubator. One hundred µl of the SOC media was plated out onto Luria Bertani Agar plates (Merck, C147) containing Ampicillin (Roche, 91084620), 5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside (X-gal) (Roche, 49234720) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Roche, 92357421) and the plates incubated at 37°C overnight (Appendix 2).

pGEM-T Easy contains the *lac*Z gene which codes for the enzyme β-galactosidase. This enzyme metabolises lactose into galactose and glucose (Stanier *et al*., 1970). β-galactosidase is also able to metabolise substrates such as X-gal, a colourless modified sugar, into a blue coloured product. IPTG, a lactose analogue, acts as a gene activator for transcription and enzyme production. Within the *lac*Z gene in pGEM-T Easy is a multiple cloning site with T-overhangs into which PCR products with A-overhangs (produced as a result of *Taq* polymerase) can be ligated. A DNA insert into this cloning site disrupts the LacZ gene and enzyme activity resulting in the non-production of β-galactosidase and resulting in white bacterial colonies. The non-disruption of the gene (no DNA insert) results in an intact and functional gene that with the expression of β-galactosidase results in blue colony formation (Sambrook *et al*., 1989).

3.2.3.7 Plasmid Preparation from Transformed Cells

Ten white colonies (clones) were selected and each colony inoculated into sterile test tubes containing 5ml Luria broth. Test tubes were incubated at 37°C with shaking at 150rpm overnight in a Labcon shaking incubator. In order to analyse the different inserts that had been ligated into the pGEM-T Easy vector that most likely represented different species within the total genomic extractions, plasmid preparations were carried out using a QIAprep Miniprep Kit (Qiagen, 27104). Plasmid preparation was carried out as per manufacturer's instructions with buffers utilised supplied with the QIAprep Miniprep Kit.

One ml of the overnight bacterial culture was aliquoted into a sterile microcentrifuge tube and bacterial cells harvested by centrifugation at 13,000rpm for 5 minutes in an Eppendorf 5415 D centrifuge. The supernatant was discarded and the pellet re-suspended in 250µl buffer P1 (re-suspension buffer). Once re-suspended, 250µl buffer P2 (lysis buffer) was added and the sample gently mixed by inversion 6 times. 350 µl of buffer N3 (neutralisation buffer) was then added and the tube inverted a further 6 times before being immediately centrifuged for 10 min at 13,000 rpm. The resulting supernatant was transferred to a QIAprep spin column and the column centrifuged for 60 seconds. The flow-through was discarded and 0.5ml buffer PB (binding buffer) added to the column. The columns were centrifuged for 60 seconds at 13,000rpm and the flow-through discarded. The columns were washed by adding 0.75ml buffer PE (wash buffer) and centrifuging for 60 seconds. The flow-through was discarded and the columns centrifuged for an additional 60 seconds to remove residual wash buffer. To elute the DNA, the QIAprep column was placed into a fresh 1.5ml microcentrifuge tube and 50µl sterile distilled water added to the centre of the column. The columns were left to stand for 2 minutes before being centrifuged at 13,000rpm for 60 seconds. The eluted plasmid DNA was stored at -20°C.

3.2.3.8 Cycle Sequencing of Cloned PCR Products

Individual PCR products ligated into pGEM-T were cycle sequenced using the BigDye V3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, 4337455) as per manufacturer's instructions. Inserts were cycle sequenced using the SP6 primer (Yalamanchili *et al.*, 1996). 20µl reactions were set up in sterile 100µl PCR tubes consisting of 4µl Big Dye Mix, 4µl 5x Buffer, 2µl SP6 primer (10µM), 6µl plasmid DNA and 4µl distilled water (Appendix 2). Cycle sequencing reactions were carried out using a GeneAmp 9700 PCR Thermocycler. An initial denaturation step at 96°C for 1 second was followed by 25 cycles of 10 seconds denaturation at 96°C, 5 seconds annealing at 50°C and 4 minutes elongation at 60°C.

3.2.3.9 Purification of Extension Products

Extension products generated from cycle sequencing reactions were purified according to the protocol provided in the Applied Biosystems BigDye V3.1 Terminator Cycle Sequencing Kit manual (Applied Biosystems, 2002).

Extension products were purified by way of alcohol precipitation. 6µl 125 mM EDTA (Sigma-Aldrich, E9884-100G) and 70µl 100% ethanol (Merck, 223 35 10 CC) was added to each extension product and incubated at room temperature for 15 minutes (Appendix 2). Samples were centrifuged in an Eppendorf 5810 R centrifuge at 13,000rpm for 30 minutes at 4°C. The supernatant was carefully discarded, 100µl 70% ethanol added and samples centrifuged at 13,000rpm for a further 15 minutes at 4°C. The supernatant was once again carefully discarded. PCR tubes were placed in an open GeneAmp 9700 PCR Thermocycler with their lids open and pellets dried at 95°C for 5 minutes in the dark. Dried pellets were stored at 4°C.

3.2.3.10Automated DNA Sequencing

Extension products were sequenced using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, 903565). Prior to sequencing, extension products were re-suspended in 10µl deionised formamide, vortexed and briefly centrifuged at 13,000rpm. Samples were heated to 95°C for 2 minutes to denature samples before being placed on ice prior to sequencing.

3.2.3.11 Organism Identification

Sequences generated from the ABI Prism 3100 Genetic Analyser were read using the BioEdit v7.07 Sequence Alignment Editor. Sequences were copied into text format and organism identities derived using the Basic Local Alignment Search Tool (BLAST) available online from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. Sequences yielding organism matches of over 95% similarity were selected and sequences yielding matches of over 98% were accepted as being species specific.

3.2.3.12 Phylogenetic Tree Calculation

Sequences generated were aligned using the CLUSTAL X Multiple Sequence Alignment program (EBI) and a phylogenetic tree generated using Treeview (Page, 1996). Phylogenetic tree graphics were finalised using CanvasX (ACD systems). Phylogenetic trees generated illustrated relationship similarities between cloned sequences generated from treatment genomic extractions and organisms present within the NCBI database. Phylogenetic trees were rooted with the 16S rDNA sequence of the archaea *Pyrococcus horikoshi* OT3.

3.2.4 Molecular Characterisation of the Fungal Populations within Treatment Rhizospheres

The fungal populations in the rhizosphere of *C.dactylon* growing in HC in pot trials was also investigated using molecular techniques.

3.2.4.1 Genomic Extraction

Genomic extractions were undertaken on 1g samples from each pot treatment harvested at the interface of the HC and cladding using the method previously described (Section 3.2.3).

3.2.4.2 Polymerase Chain Reaction Amplification of Fungal Internal Transcribed Spacer Regions

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA are highly variable sequences of great importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz, 2005). Organisms present in each treatment were identified by their rDNA sequences that were PCR amplified. The primer set of ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990) were utilised with these primers primarily amplifying both ITS regions of fungal ribosomal DNA as well as the 5.8S subunit separating them (Martin and Rygiewicz, 2005).

PCR reactions (25µl) mirroring reactions set up for mycorrhizal species characterisation (3.2.3.3) were established however, 2µl 5mM ITS1-F primer and 2µl 5mM ITS4 primer were added to each reaction instead.

PCR reactions were carried out in an Applied Biosystems GeneAmp 9700 PCR Thermocycler. An initial denaturation step at 94°C for 2.5 minutes was followed by 40 cycles of 15 seconds denaturation at 94°C, 30 seconds annealing at 53°C and 90 seconds elongation at 72°C. A final elongation step at 72°C for 10 minutes completed the reaction.

PCR products obtained were further cloned and sequenced as per protocol described for mycorrhizal fungal identification (Sections 3.2.3.4 to Section 3.2.3.12). After transformation, 5 white colonies (clones) from each treatment were selected for sequencing (A-E, Figure 3.4).

3.3 Results

3.3.1 Most Probable Propagule Number

Using the Most Probable Propagule determination method, it was calculated that the mycorrhizal inoculum utilised in trials contained 64 viable propagules per gram of inoculum.

3.3.2 Morphological Characterisation of the Mycorrhizal Inoculum

Microscopic analysis of spores extracted from the mycorrhizal inoculum utilised in trials revealed spores of varying morphologies (Figure 3.1). The majority of spores extracted from the mycorrhizal inoculum were approximately 100µm in size though a distinct type of spore, bright yellow in colour (Figure 3.1, D), was readily encountered and noted as being slightly larger in size than the other spores observed. Spores were generally brick red to brown/yellow in colour and the majority of spores had 2 to 3 (**x**) cell wall layers. Clear hyphal attachment (*) could be distinguished on most spores (Figure 3.1, A-C).

Species identification of extracted spores based on INVAM literature descriptions was not found to be accurate, primarily due to a lack of cell wall clarity when specimens were analysed using light microscopy. Sample preparation most likely contributed to this factor. However, from spore size calculations and hyphal attachment, the majority of spores extracted from the inoculum were considered to belong to the genus *Glomus*. Molecular characterisation techniques were thus utilised to more accurately identify AM fungal types within the inoculum to the species level with a greater degree of confidence.

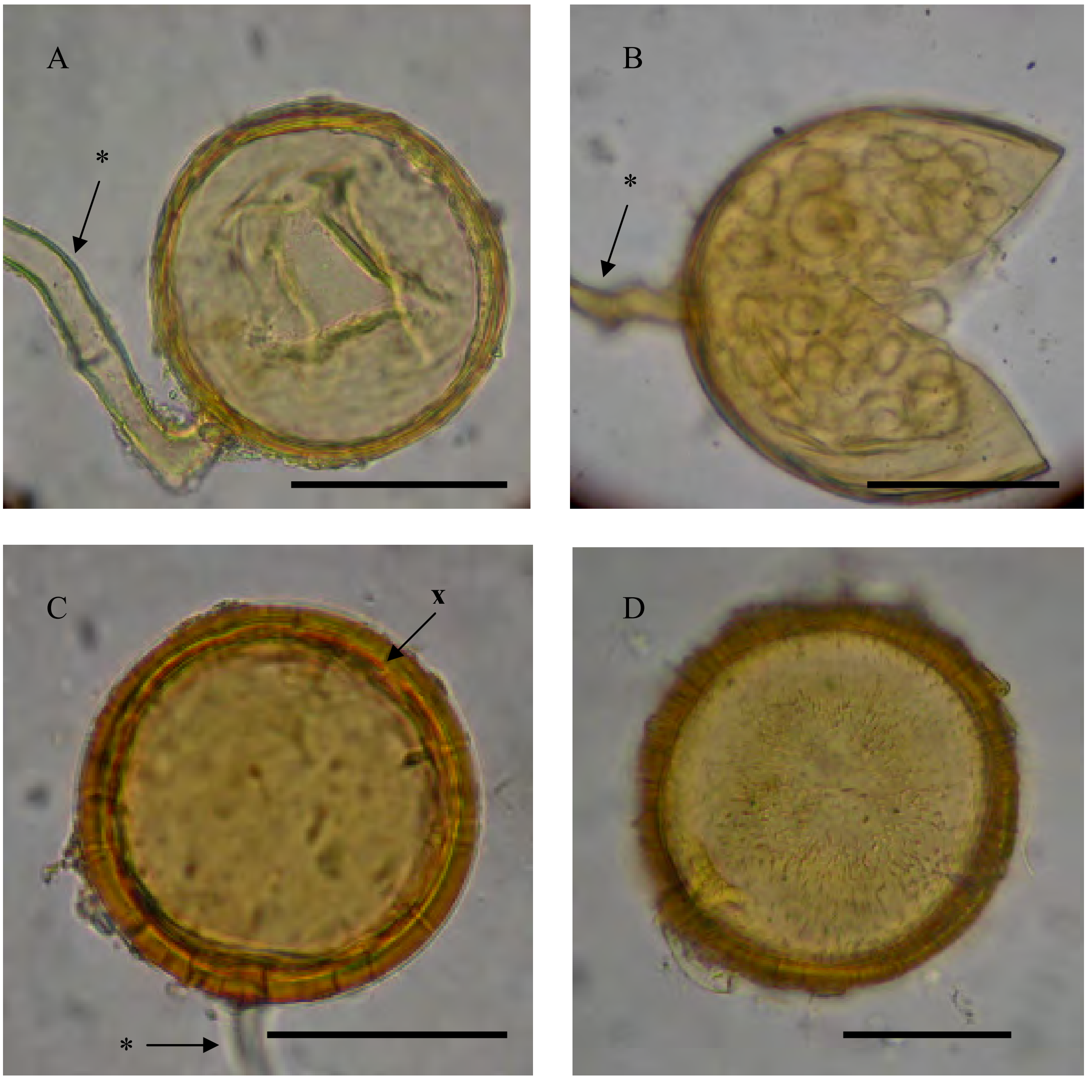

Figure 3.1. Micrographs of various spore types isolated from the mycorrhizal inoculum utilised in this study. Spores of varying morphologies were observed from the mycorrhizal inoculum used with the majority having hyphae attached. The majority of spores appeared to have characteristics indicative of the *Glomus* genus. Scale Bar = 50µm. Two to three cell wall layers (x) and hyphal attachments (*) were frequently observed on the spores examined.

3.3.3 Molecular Characterisation of the Mycorrhizal Inoculum

PCR amplification of extracted genomic DNA from the mycorrhizal inoculum resulted in correctly sized bands of ~560bp in size. Analysis of the sequences revealed the presence of 4 mycorrhizal species: *Glomus clarum* (*G.clarum*)*, Gi.gigantea, G.mosseae* and *Paraglomus occultum* (*P.occultum*), formerly *Glomus occultum* (*G.occultum*) (Morton and Redecker, 2001). Sequence identity percentages of 95%, 95%, 95% and 96% were obtained for each sequence respectively. A phylogenetic tree depicting the mycorrhizal species identified from BLASTED clones in relation to the most closely related organisms (NCBI) is illustrated in Figure 3.2.

Molecular characterisation results of the species present within the mycorrhizal inoculum confirmed initial conclusions made from morphological analysis with 2 *Glomus* species being identified. Spores belonging to the genus *Paraglomus* may have not been morphologically identified due to a lack of sporulation by this genus at the time of analysis

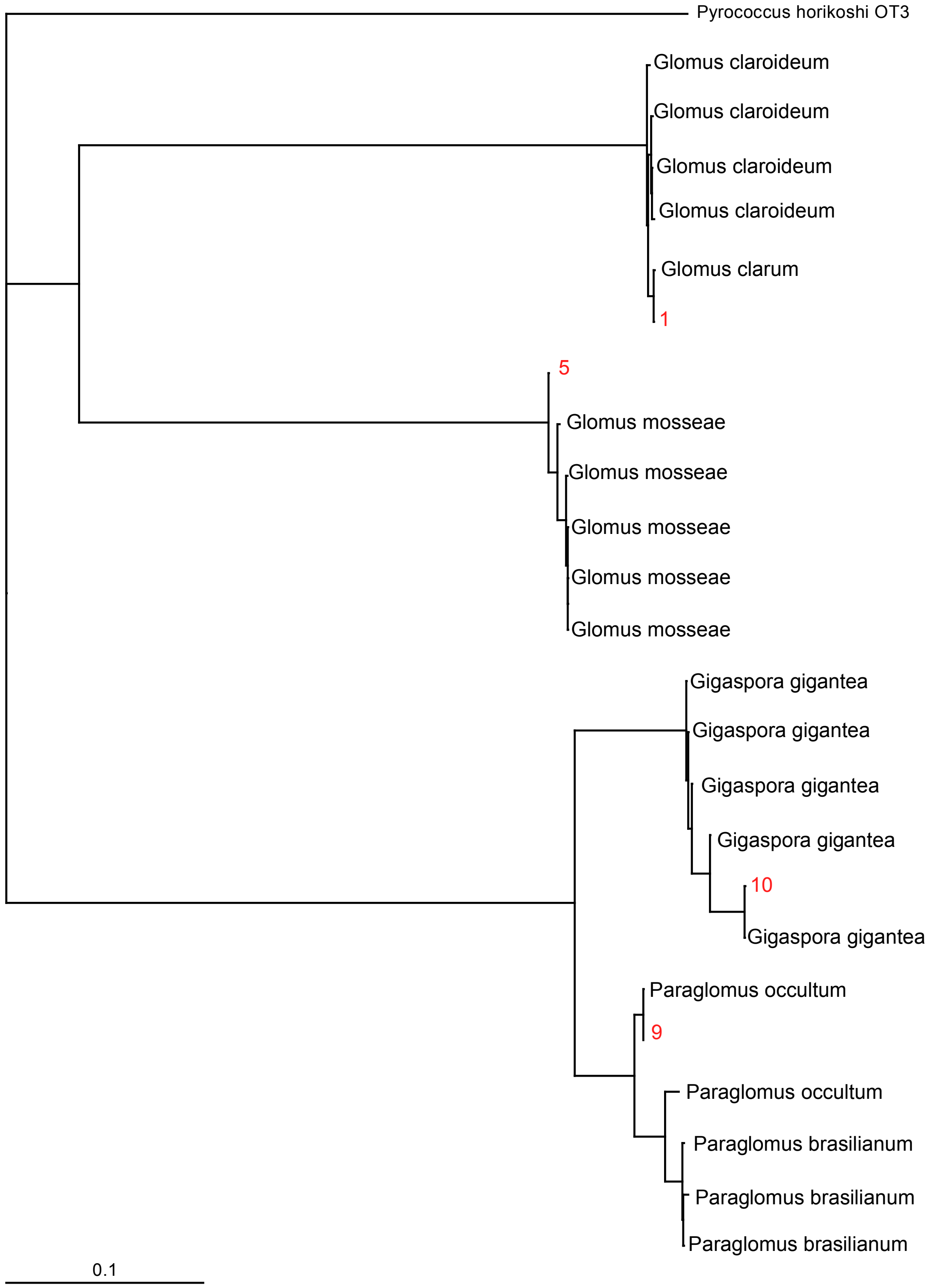

Figure 3.2. Rooted phylogram illustrating species of arbuscular mycorrhizal fungi present in the inoculum utilised in trials. Numerals reference the clone numbers that were sequenced and BLASTED from which identity matches were obtained.

3.3.4 Molecular Characterisation of the Fungal Populations within Treatment Rhizospheres

PCR amplification of extracted genomic DNA utilising the ITS1-F and ITS4 primer pair produced correctly sized bands (Figure 3.3) of around 580bp in size (Gardes and Bruns, 1993). Subsequent separation and sequencing of individual bands (clones) to identify rhizospheric organisms present within the coal substrate of pot trials showed a dominance of species belonging to the genera *Trichoderma*, *Penicillium* and *Aspergillus*. The phylogenetic tree constructed using the complete number of clones BLASTED from each treatment that resulted in matches of over 95% to the closest related organisms (NCBI) is depicted in Figure 3.4.

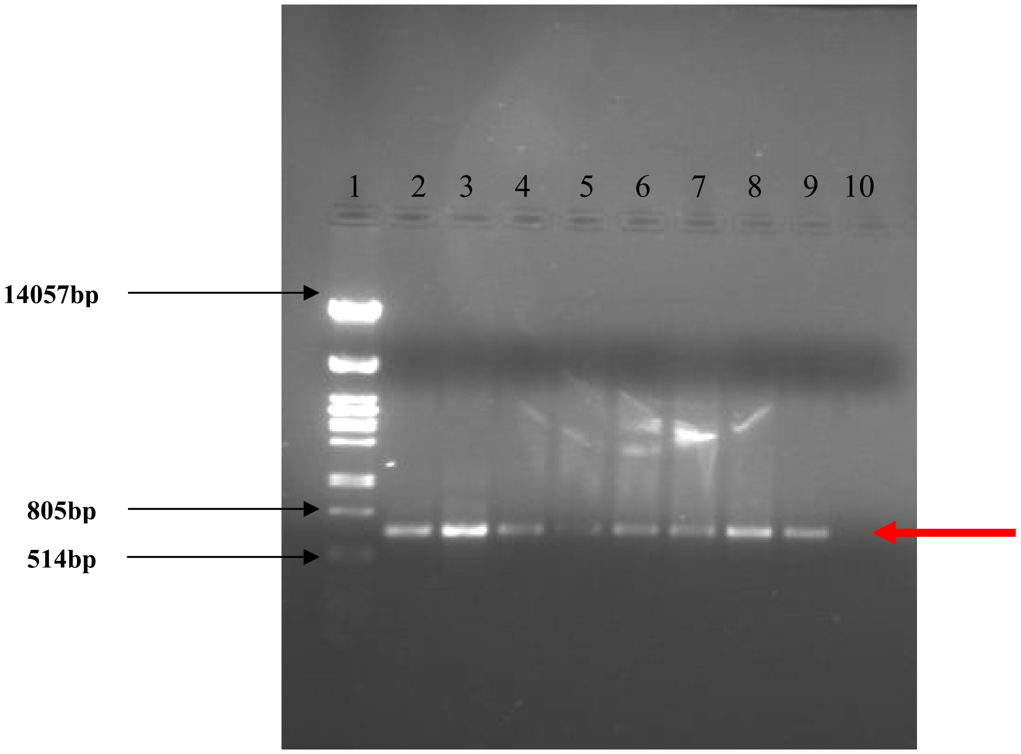

Figure 3.3. Agarose electrophoresis gel of PCR products derived from ITS1-F/ITS4 primer amplification of extracted genomic DNA across all treatments. Lane 1: λ *Pst* Molecular Marker, Lane 2: Hard Coal + Weathered Coal + *Cynodon dactylon*, Lane 3: Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Lane 4: Hard Coal + Weathered Coal + *Cynodon dactylon* + EBRU Culture Collection Isolate Number 84, Lane 5: Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84, Lane 6: Hard Coal + Soil + *Cynodon dactylon*, Lane 7: Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Lane 8: Hard Coal + Soil + *Cynodon dactylon* + EBRU Culture Collection Isolate Number 84, Lane 9: Hard Coal + Soil + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and Lane 10: Negative Control.

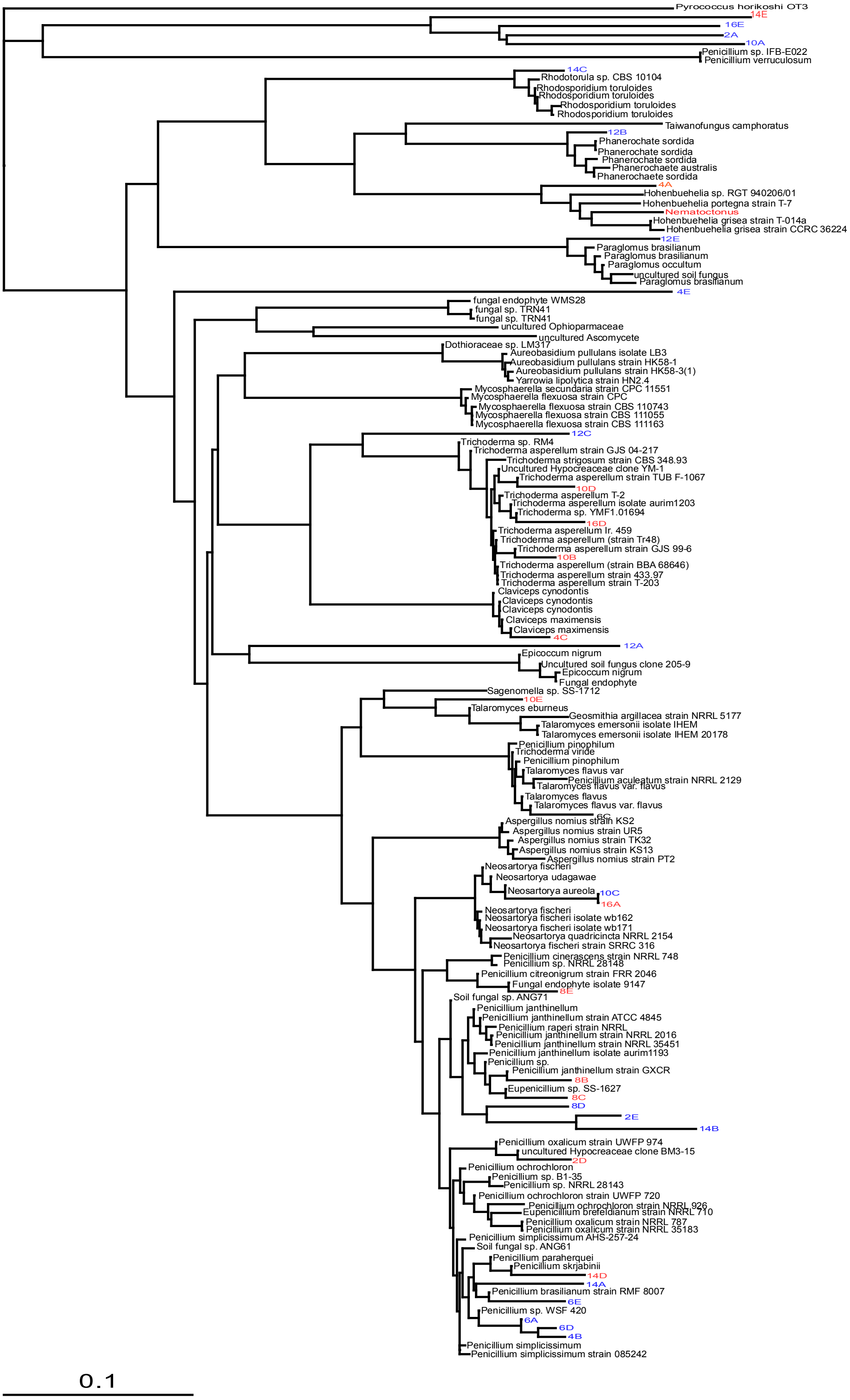

Figure 3.4. Rooted phylogram illustrating organisms in samples of the rhizosphere of *Cynodon dactylon* growing on hard coal amended with a weathered coal supplement (Red) and those cladded with soil (Blue). Numerals reference the clone numbers from various treatments that were sequenced and BLASTED from which identity matches were obtained. The treatments to which each numeral refers to are listed in Table 3.1 and Table 3.2.

Population studies of treatments involving the growth of *C.dactylon* on HC amended with a WC supplement revealed the presence of a wide variety of fungal genera with *Penicillium* being readily present (Table 3.1). For the control treatment (HC + WC + *C.dactylon*), *Penicillium oxalicum* (*P.oxalicum*) and *Mycosphaerella flexuosa* (*M.flexuosa*) were identified in the coal substrate. In the treatment HC + WC + *C.dactylon* + AMF, a *Penicillium* species was once again identified together with a *Nematoctonus* species, *Claviceps cynodontis* (*C.cynodontis*) and *Taiwanofungus camphorates* (*T.camphorates*). The treatment HC + WC + *C.dactylon* + ECCN84 showed the presence of 3 *Penicillium* species, including *Penicillium skrjabinii* and *Penicillium janthinellum* (*P.janthinellum*). Species of *Rhodotorula* and *Trichoderma* were also identified from this treatment. In the remaining treatment HC + WC + *C.dactylon* + AMF + ECCN84, *N.fischeri* was re-isolated from the coal substrate together with *Trichoderma asperellum* (*T.asperellum*) and *Aureobasidium pullulans* (*A.pullulans*).

Analysis of the rhizospheric organisms present within trials where HC was cladded with soil also showed that *Penicillium* species were readily present (Table 3.2). Where *C.dactylon* was grown solely (HC + Soil + *C.dactylon*), 4 separate *Penicillium* species were isolated including *Penicillium pinophilum* (*P.pinophilum*), as well as *Aspergillus nomius* (*A.nomius*). *Penicillium* species once again dominated in the treatment HC + Soil + *C.dactylon* + AMF with *P.janthinellum, Penicillium raperi* (*P.raperi*) and *Penicillium citreonigrum* (*P.citreonigrum*) isolated. In the treatment HC + Soil + *C.dactylon* + ECCN84, *N.fischeri* was re-isolated alongside a species of *Penicillium* and *Talaromyces eburneus* (*T.eburneus*). The only mycorrhizal species isolated, *P.occultum*, was identified in the treatment HC + Soil + *C.dactylon* + AMF + ECCN84 alongside a fungal endophyte, *Phanerochaete sordida* (*P.sordida*) and *T. asperillum*.

Table 3. 1. Fungi in the rhizosphere of *Cynodon dactylon* growing on hard coal amended with a WC supplement. Percentage homologies across amplified sequences with species within the NCBI database are bracketed.

Treatment	**Species Identified**
HC + WC + *C.dactylon* (Clone Numbers 2)	*Mycosphaerella flexuosa* (98) *Penicillium oxalicum* (99) Soil fungal sp. (95)
HC + WC + *C.dactylon* + AMF (Clone Numbers 4)	*Nematoctonus sp.* (98) *Penicillium sp.* (95) *Claviceps cynodontis* (98) *Taiwanofungus camphorates* (98)
HC + WC + *C.dactylon* + ECCN84 (Clone Numbers 14)	*Penicillium skrjabinii* (98) *Penicillium janthinellum* (98) *Rhodotorula sp.* (96) *Penicillium sp.* (98) *Trichoderma sp.* (97)
HC + WC + *C.dactylon* + AMF + ECCN84 (Clone Numbers 16)	*Neosartorya fischeri* (98) *Trichoderma asperellum* (98) *Aureobasidium pullulans* (98)

Table 3. 2. Fungi in the rhizosphere of *Cynodon dactylon* growing on hard coal cladded with soil. Percentage homologies across amplified sequences with species within the NCBI database are bracketed.

Treatment	**Species Identified**
HC + Soil + *C.dactylon* (Clone Numbers 6)	*Penicillium sp.* (95) *Penicillium pinophilum* (98) *Penicillium sp.* (95) *Penicillium sp.* (95) *Aspergillus nomius* (98)
HC + Soil + *C.dactylon* + AMF (Clone Numbers 8)	*Penicillium janthinellum* (98) *Penicillium raperi* (100) Unknown soil fungal sp. (95) *Penicillium citreonigrum* (98)
HC + Soil + *C.dactylon* + ECCN84 (Clone Numbers 10)	*Penicillium sp.* (95) Uncultured Hypocreaceae (97) *Neosartorya fischeri* (98) *Talaromyces eburneus* (100)
HC + Soil + *C.dactylon* + AMF + ECCN84 (Clone Numbers 12)	*Fungal endophyte* (96) *Phanerochaete sordida* (96) *Trichoderma asperellum* (96) *Paraglomus occultum* (98)

3.4 Discussion

3.4.1 Molecular Characterisation of the Mycorrhizal Inoculum

The presence of AMF species on coal mining sites has been well documented in a variety of studies (Mehrotra, 1998; Melloni *et al.*, 2003; Ning and Cumming, 2001). As previously described, the main beneficial effect of AMF is that of enhanced P uptake (Smith and Read, 1997). However, studies carried out have suggested that mycorrhizal plant establishment is not only enhanced due to increased P acquisition, but that mycorrhizal fungi also enhance the uptake of other elements (Leake and Read, 1989; Pacovsky, 1986). Therefore, the maintenance in balance of a wide variety of nutrients could most likely be key in plant survival rather than just the sole role of enhanced P uptake (Ning and Cumming, 2001). The regulation of nutrient uptake in plants is a process that, naturally, will affect nutrient balances within the plant (Marschner, 1995). It is this balance in nutrient acquisition modulated by mycorrhizal fungi by which plants under soil stress are able to survive. Lux and Cumming (2001) demonstrated that the reduction of Al toxicity in *Liriodendron tulipifera* L. by mycorrhizal fungi was not only enhanced by P uptake, but also by the balanced acquisition of Ca, Cu and Zn in roots and shoots. Simultaneously, mycorrhizal fungi can also suppress the uptake of elements such as Al, Fe and Mn that in some soils may be present at toxic levels (Kothari *et al.*, 1990).

Molecular characterisation of mycorrhizal fungal species present within the coal inoculum utilised revealed the presence of the species *G.clarum, G.mosseae, P.occultum* and *Gi.gigantea* (Figure 3.2). Mycorrhizal species belonging to the *Glomus* genus have often been isolated in association with plants growing on lands disturbed by coal mining activity. In a study carried out by Bi *et al.* (2003), the determination of whether mycorrhizal colonisation could promote the growth of maize and enhance its utilisation of nutrients when grown over coal fly ash was investigated. From this study it was revealed that inoculating plants with *G.mosseae* increased their growth in comparison to non-mycorrhizal plants. This enhanced growth was due to the mycorrhizal plants absorbing more nutrients than non-mycorrhizal plants and this reduced the translocation of Na to the plant shoots, with salt accumulation being higher in non-mycorrhizal plants. Further, a variety of studies investigating the potential of AMF to enhance plant growth in areas of coal mining have been based on tolerance to high soil Al levels present at these sites. Klugh and Cumming (2005) investigating the detrimental effects of Al to plant physiology demonstrated that plants display a wide range of sensitivity to Al in the rhizosphere with organic acid exudation being key to Al resistance. Organic acids are able to chelate Al rendering it non-toxic to plants (Zheng *et al.*, 1998). The study revealed that *Andropogon virginicus* (L.) (broomsedge), an early successional species that colonises disturbed mining areas, is able to resist Al toxicity with AMF influencing organic acid exudation from this plant. In comparison to non-mycorrhizal plants, a variety of mycorrhizal species associated with the plant roots including *G.clarum* enhanced the production of citrate and malate organic acids that were implicated in Al resistance (Kelly *et al.*, 2005). *G.occultum* has readily been found associated with plants re-colonising mine soils (Morton, 1985) as well as overburden material from abandoned coal mines (Wright *et al.*, 1987). The fourth species of mycorrhizal fungi isolated from the coal inoculum was that of *Gi.gigantea*. This species of mycorrhizal fungi has been isolated from anthracite waste and subsequently suggested as an agent to help re-vegetate coal mine spoils (Daft and Hacskaylo, 1976) with the ability of the fungus to enhance P nutrition and maintain nutrient balances within plants most likely being key to plant establishment on such discard material (Ning and Cumming, 2001).

Should mine spoils be effectively rehabilitated utilising mycorrhizal fungi, it is important to identify which fungi are most likely to be favoured in a particular environment as well as with which specific plant species (Mehrotra, 1998). It has been demonstrated that different mycorrhizal isolates confer different levels of enhanced nutrient uptake by the plant with coal spoil conditions affecting this ability as well as colonisation rates (Kahn, 1981). Key sources of mycorrhizal inoculum containing strains well adapted to discard coal conditions could therefore be derived from discard mine spoils containing established vegetation (Danielson, 1985). The species of mycorrhizal fungi identified from the discard coal dump in Navigation most likely were adapted to rhizospheric conditions within the dump as they were readily present in the roots of plants growing on these dumps. These species of mycorrhizal fungi would have further conferred to the plant such benefits described as enhanced nutrient uptake, nutrient homeostasis and increased tolerance to possible heavy metals within the discard coal. The inoculation of plants with the mycorrhizal isolates identified could therefore enhance plant establishment on discard coal dumps and lead to efficient re-vegetation of disturbed landscapes.

3.4.2 Molecular Characterisation of the Fungal Populations within Treatment Rhizospheres

Fungi are a very diverse group of organisms and encompass a wide range of forms. Most fungal species spend a portion of their life-cycle either within or linked to the soil environment with their role in this environment being complex, and also one that is vital to the overall rhizospheric ecosystem (Wainwright, 1998; Bridge and Spooner, 2001).

The limitations of morphological techniques used to identify fungi has led to a preference for molecular techniques in fungal classification and identification (Bruns *et al.*, 1991). The development of total genomic extraction and analysis techniques have enabled studies of complex environmental samples (Bridge and Spooner, 2001). Molecular characterisation of fungi has largely focussed on the rDNA gene cluster composed of a structured unit consisting of three ribosomal rDNA subunit genes and internally transcribed spacer regions (Martin and Rygiwiecz, 2005). Due to the DNA sequences within the subunits containing some extremely conserved sequences, broad specificity primers can be developed including some that are specific for fungi in general or are even phylum specific (Bruns *et al.*, 1990). Utilisation of these primers can therefore allow for the amplification of fungal DNA from samples containing nucleic acids from other phyla as well (Gardes and Bruns, 1993). The one significant limiting factor in studying soil fungi by molecular techniques is the lack of differentiation between living and dead fungal material as well as active and dormant organisms (Bridge and Spooner, 2001).

Analysis of the substrate in pots containing *C.dactylon* growing on HC amended with a WC supplement revealed a heavy presence of Ascomycetous fungi with *Penicillium* and *Trichoderma* species pre-dominating (Table 3.1). These two genera, especially *Penicillium*, are widely present in soils (van Elsas *et al.*, 1997). Further identified were the plant pathogens *M.flexuosa* (Hunt *et al.*, 2006), *C.cynodontis*, a widespread parasite of *C.dactylon* (Pažoutová *et al.*, 2005), the chitinolytic and xylanolytic fungus *P.janthinellum* (Di Giambattista *et al.*, 2001), the mycoparasitic fungus *T.asperellum* (Ramot *et al*., 2004), *P.oxalicum*, a biocontrol agent of *Fusarium* (Larena *et al*., 2002), the dematiaceous and occasionally human pathogenic fungus *A.pullulans* (Hawkes *et al*., 2005) and interestingly, the species *T.camphorates,* a relatively new genus identified in Taiwan (Wu *et al*., 2004). Most importantly, *N.fischeri* was also identified from the treatment HC + WC + *C.dactylon* + AMF + ECCN84. This demonstrates the recovery of an organism in the inoculum.

The initial isolation of this organism from the *C.dactylon* rhizosphere on coal dumps, its introduction into the pot trial inoculum and its recovery after the experimental period fulfilled 3 of the steps required to demonstrate Koch's postulates following the 22 week growth study. In qualifying this observation, it should be noted that components of the original spore inoculum rather than new growth of ECCN84 would need to be demonstrated to confirm the Koch's postulate observations. However, this seems less likely as Igbinigie (2007) did confirm the presence of *N.fischeri* within coal substrate sampled from the Navigation Mine site suggesting its presence within Kroomdrai coal samples as well. The provisional assumption was made that recovery of the organism correlated to an enhanced inoculation within the pot study system. Further studies utilising sterile substrate would confirm the postulates.

Similar fungal species were identified in the pot study involving HC cladded with soil. Once again, *Penicillium* and *Trichoderma* species were present in all samples (Table 3.2). Selected species which had been identified in the pot trials with HC amended with a WC supplement were once again observed together with a variety of soil-borne fungi such as *T.eburneus* (Yaguchi *et al*., 1994), *P.raperi* and *P.citreonigrum* (Nesci *et al*., 2006), the soil dwelling and aflatoxin-producing *A.nomius* (Ehrlich *et al*., 2007), the mycoparasitic *P.pinophilum* (De Stefano *et al*., 1999), the widely distributed ligniloytic white-rot fungus *P.sordida* (Lim *et al*., 2003) and the mycorrhizal fungus *P. occultum* (Ferrol *et al*., 2004).

It is interesting that only one species of AMF present in the trial inoculum, *P.occultum*, was re-isolated in the pot trial after the growth period. This contrasts strongly with high colonisation rate observed in roots examined and may support the observation that extraradical hyphal establishment is reduced in a HC rhizosphere, more so where WC is utilised as a supplement in comparison to soil. To confirm the role played by AMF species in the growth of *C.dactylon*, species present in the inoculum, analysis of the species colonising the plant roots alongside species present in the rhizosphere would need to be identified as well. Due to the nature of the association, it is likely that this would result in the identification of the alternate inoculum AMF species as well. Though AM fungal species within the rhizosphere would have been amplified by the ITS1-F/ITS4 primer pair, utilisation of the AMF specific AM1/NS31 primer pair alongside may have been a more accurate method of measuring rhizospheric mycorrhizal populations.

Analysis of fungal species within pots revealed a heavy dominance of *Penicillium* species within the coal substrate. Characterisation of soil microorganisms often results in the isolation of *Penicillium* and *Aspergillus* species mainly due to the fact that these fungi sporulate profusely and thus are able to establish significant populations (Alexander, 1967). Similar identifications have been made when identifying fungal species from discard coal dumps. Studies characterising bacterial and fungal species present within discard coal dumps have mainly focussed on screening for species that may have the ability to solubilise coal. Pokorný *et al.* (2005) on characterising microorganisms isolated from lignite excavated from the Záhorie coal mine in south-western Slovakia revealed a heavy dominance of the genera *Trichoderma*, *Penicillium*, *Epicoccum*, *Metarhizium* (*Cordyceps*), and *Cladosporium* according to ITS sequence analysis. Similar genera, mainly *Trichoderma* and *Penicillium* were identified within the HC utilised in pot trials. The species isolated from the coal substrate in pot trials also mirrored species identified by Igbinigie (2007) as being present within the discard HC dumps at the Navigation Mine site where initial observations were made.

A number of studies have analysed the coal-solubilising potential of a variety of *Penicillium* and *Trichoderma* species (Hölker *et al.*, 2002; Achi, 2004; Hölker and Höfer, 2004). However, of the fungi identified within the coal substrate of different treatments, species belonging to the genus *Phanerochaete* have readily been shown to have coal-degrading capabilities, including *Phanerochaete chrysosporium* which has been well documented (Houtkoop-Steenstra *et al.*, 1997; Ralph and Catcheside, 1998; Ralph and Catcheside, 1999). A further fungal species identified in certain treatments that has been shown to have coal solubilising ability was *Neosartorya fischeri* (Igbinigie *et al.*, 2007) referred to as ECCN84 in this study. This species was identified from treatments that were directly inoculated with the fungus and it therefore most likely played a role in the results observed from these treatments.

3.5 Conclusions

- The AM fungal species present within *C.dactylon* plants growing on discard HC dumps at the Navigation Mine site are *P.occultum, G.clarum, G.mosseae* and *Gi.gigantea*. These species of AMF may have played a key role in plant establishment and most likely have contributed towards the biological soil formation observed. The recovery of only 1 AMF inoculum species within treatment rhizospheres further suggested an influence on extraradical hyphal establishment by the HC environment.

- Non-mycorrhizal fungal species identified within pot trial substrate material were similar to species commonly encountered in discard HC dumps as well as species identified by Igbinigie *et al.* (2007) as being present at the Navigation Mine site HC discard dumps.

- The re-isolation of ECCN84 from treatment rhizospheres fulfilled 3 of Koch's postulates and indicated its influence on results obtained.

CHAPTER 4:

SIMULATION OF THE BITUMINOUS HARD COAL DISCARD DUMP ENVIRONMENT 1: STRUCTURAL CHANGES

4.1 Introduction

Observations at the Anglo Coal Navigation Mine site had revealed that alongside the sporadic growth of *C.dactylon* on un-rehabilitated HC dumps was the formation of a humic soil-like material from the coal. Initial observations suggested that this process was due to the action of the plants and associated mycorrhizal and non-mycorrhizal rhizospheric microbial populations. Subsequent pot studies revealed that substantial *C.dactylon* growth on HC was obtained when it was amended with an oxidised WC supplement. Further, *C.dactylon* plants grown in association with particular AMF and ECCN84 inocula produced the highest biomass yields. Pot trials to some extent clarified the role played by both mycorrhizal and non-mycorrhizal rhizospheric fungi in the establishment of *C.dactylon* on HC.

Given the complexity of the natural dump environment, the pot trials were considered to be an over simplification of this system and an attempt was made to set up a simulation of the *C.dactylon*/coal/AMF/rhizospheric fungal system. To enable the investigation of these questions under more realistic yet controlled laboratory conditions, column reactors were designed to replicate the profile of the upper 150cm of the dump environment. *C.dactylon* was planted into the top of these columns, the coal substrate was inoculated with AMF and ECCN84 and changes effected within the rhizospheric coal substrate, particularly reflecting coal biotransformation, were followed.

4.2 Methods

4.2.1 Column Trial Set-Up

Columns of 150cm were constructed of PVC guttering with a front face of clear Perspex (Figure 4.1 and 4.2). The front of the column was fitted with a rubber gasket to ensure it was air and water tight and the front face was tightly screwed onto the column (Figure 4.3). The bottom of the column was fitted with a tap to allow for drainage (Figure 4.4). Prior to initiation of the trial, columns were surface sterilised with 50% sodium hypochlorite solution to reduce contaminants from entering the experimental system. During growth, columns were covered with black plastic sheeting to prevent growth of photosynthetic microorganisms and to replicate the dark conditions of the rhizosphere (Figure 4.5).

HC collected from the Anglo Coal Kroomdraai Mine (Witbank, South Africa), of a known size fractions, was used to pack the column to a depth of 120cm and to the top 20cm of the column was added a 25% WC supplement, also of known size fractions. For comparative HC controls, the WC supplement was excluded from the upper layer of the column. *C.dactylon* seedlings were planted into each column with the exception of the controls. *C.dactylon* seed (0.5g) was initially planted in soil in individual sections (0.2cm x 0.2cm) of a surface sterilised styrofoam seedling tray. Seedlings were allowed to germinate and grow for a period of 1 week prior to single plugs being transplanted to the centre of the WC supplement layer in each column.

For treatments requiring inoculation with AMF, 23g of mycorrhizal inoculum (as utilised in the pot trials) was added to each column. This was distributed evenly and lightly covered with WC supplement material. For treatments requiring the addition of ECCN84, 100ml of a spore suspension (70,000 spores/ml) was poured evenly into each column (prepared as in pot trials). Columns were held upright in a constant environment chamber set at a temperature of 25°C with a day:night light regime of 16:8 hours provided by banks of Grolux fluorescent tubes linked to an automated timer (Figure 4.5). Incident light was approximately 150µmoles/m^2/sec. Each pot was watered daily with 100ml tap water.

The experiment consisted of the following treatments:

1. HC + WC Supplement (+*C.dactylon*, -AMF, -ECCN84)
2. HC + WC Supplement (+*C.dactylon*, +AMF, -ECCN84)
3. HC + WC Supplement (+*C.dactylon,* +AMF, +ECCN84)
4. HC (Control)

+ : denotes presence
- : denotes absence

For each treatment, 2 columns were set up (8 columns in total) and columns were harvested and analysed after 44 weeks.

Figure 4.1. Frontal view of a column used in the coal dump simulation studies. Columns were constructed out of sturdy Perspex with a front cover screwed on. The author shows the comparative size of the column with dimensions of 1.50m height, 12.7cm width and 6.7cm depth.

Figure 4.2. Side view of a column used in the coal dump simulation studies with the author once again showing the comparative size of the column.

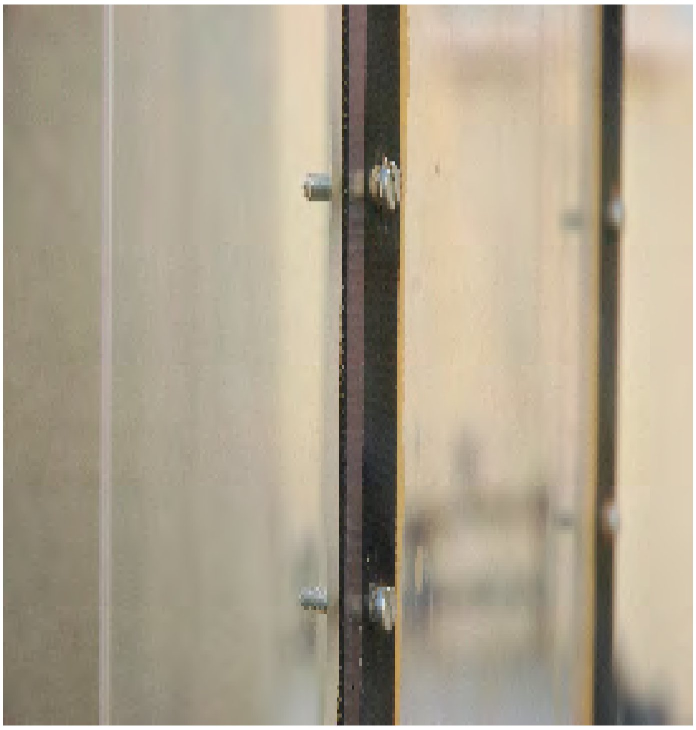

Figure 4.3. A close-up view of the Perspex front covers which were tightly screwed onto columns to ensure they were water tight, to prevent oxygen ingress and to maintain the coal substrate in place.

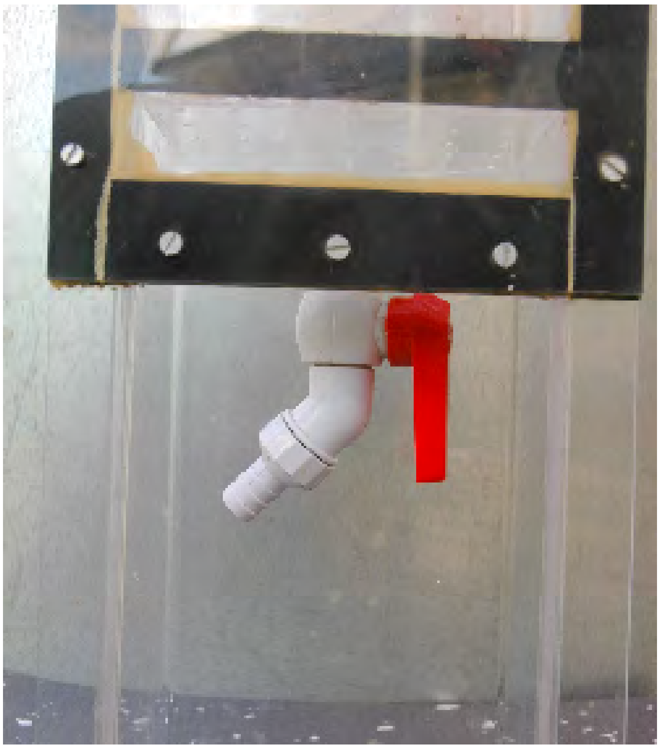

Figure 4.4. A close-up view of a tap fitted onto the base of a column to allow for adequate water drainage.

Figure 4.5. Columns used in the coal dump simulation study were placed upright and maintained in a constant environment chamber, as shown in the picture, and exposed to a temperature of 25°C and a day: night light regime of 16:8 hours. *Cynodon dactylon* was planted in the surface layer and columns watered daily.

4.2.2 Sampling

Prior to sampling, columns were laid down horizontally and the clear Perspex cover on the front of the column was removed to expose the coal substrate. Sampled material was placed in sterile sealable containers and stored at 4°C until analysed. Once samples had been obtained, the plastic cover of the column was tightly screwed back onto each column and the column returned to the constant environment laboratory.

Sampling thus consisted of replicate readings taken from two different columns. Further results obtained from material sampled from columns are discussed in chapter 5.

4.2.3 Analysis

4.2.3.1 Visual Analysis of the Coal Substrate

At harvest, the columns were examined visually and structural changes recorded photographically.

4.2.3.2 Mycorrhizal Colonisation of Cynodon dactylon Roots

To confirm the presence of AMF in *C.dactylon* roots, samples of ~ 0.5g were harvested from plants growing in HC at the top (20cm), middle (80cm) and bottom (140cm) of the columns. The roots samples were analysed as previously described for pot trials (Section 2.2.2.2).

4.2.3.3 Scanning Electron Microscopy

Coal samples were prepared for Scanning Electron Microscopy (SEM) according the methods of Cross and Pinchuck (2001).

Coal samples (~5mm in size) were harvested from the 20cm depth in the columns and were fixed overnight in 2.5% glutaraldehyde fixative solution prepared from a 25% glutaraldehyde stock reagent (Appendix 3) diluted with phosphate buffer (0.1M, pH 7.0). The glutaraldehyde solution was decanted and the coal particles washed twice for 10 minutes with phosphate buffer. Coal particles were then dehydrated by passaging through an ethanol series (30%, 50%, 70%, 80%, 90%, 100%) for 15 minutes each. The final passaging of samples in 100% ethanol (Merck, 223 35 10 CC) was carried out twice. Samples were then placed in a Polaron Critical Point Dryer for 2 hours before being mounted on 12mm diameter Al posts with 12mm graphite conducting adhesive tabs. The samples were transferred to a Balzers Union sputtering device and gold coated for 160 sec at 80 Millitorr pressure with an applied current of 45milliAmps. C paint was applied to the point of fixture to ensure a conductive path for electrons on the surface to reach ground state. Prepared samples were examined with a VEGA LMU (VEGA © Tescan) Scanning Electron Microscope.

4.2.3.4 Size Fractionation of the Coal Substrate

Size fractionation of the coal substrate was undertaken to investigate the biological degradation of coal under the influence of the various treatments. This involved the destructive sampling of a separate third column. Whole coal substrate sections were removed from the column between the 20-40cm, 70-90cm and 120-140cm column depths. The top 20cm WC supplement section of the treated columns was also harvested. Collected samples were dried at 60°C for 24 hours. Dried samples were size fractionated through a nest of sieves (apertures 8mm, 2mm, 1mm and 500µm respectively). Following sieving, the fraction in each sieve was weighed. Material passing through the last sieve into the collection pan was also weighed. A percentage weight per size fraction was calculated.

Size fraction percentages of the HC and WC supplement utilised prior to trial initiation were also measured.

4.2.3.5 Root Exudate Analysis for Organic Acids

Plastic PVC pipes 20cm long and 4.5cm in diameter were sectioned and washed in 50% sodium hypochlorite for surface sterilisation. The pipes were allowed to dry, one opening sealed with cotton mesh and the pipes 4/5 filled with an inert clay carrier. In 5 pipes, 7.5g mycorrhizal fungal inoculum and 2g *C.dactylon* seed was added on top of the carrier and lightly covered. In 5 separate pipes, 2g *C.dactylon* seed was added on top of the carrier before being lightly covered. Seeds were allowed to germinate and were left to grow for 12 months. Pipes were maintained at a temperature range of 18 to 25°C and were watered once daily with UV sterilised water and once weekly with low-P Long Ashton's Nutrient Solution (Hewitt, 1966).

Sterile deionised water (200ml) was drained through the carrier in which mycorrhizal and non-mycorrhizal plants were grown for a period of 10 hours and collected in a sterile 250ml beaker. Root growth in each plant had penetrated past the mesh containing the inert carrier within the pipes. Pipes were thus placed in the beakers containing collected water for a further 48 hours. Root exudation patterns would most likely have continued during this time and organic acids exuded into the water. Water from the differentially treated plants was collected and centrifuged at 5000rpm for 5 minutes at 4°C in an Eppendorf 5810 R centrifuge to pellet any debris and the supernatant was collected.

Organic acids present within root exudates collected were derivitised according to the methods of Villas-Boas *et al.* (2003). Root exudate sample (300µl) was collected in a sterile test tube and 50µl pyridine (Sigma-Aldrich, 360570) added to the sample. Further, 250µl of 1% sodium hydroxide (Analytical Reagents, RPCK3320) in methanol (Appendix 3) and 30µl methyl chloroformate (Aldrich, M3,530-4) were added to the sample and it was vortexed for 30 seconds. Chloroform (600µl) was added to the sample and sample vortexed for a further 10 seconds. Finally, 600µl 50mM sodium bicarbonate (Merck, 360570) was added to the sample and the sample vortexed for 10 seconds (Appendix 3). The upper aqueous layer was discarded and the lower aqueous layer was collected. Samples were filtered through a sodium sulphate column (Merck, 582 55 20 EM) and collected in a 5ml Agilent Technologies vial. Samples were stored at 4°C.

Organic acids present within the root exudates collected were identified using gas chromatography-mass spectrometry (GC-MS). Vials containing samples were placed into a 7683B Automatic Series Injector (Agilent Technologies). For each sample, about 1µl was injected into a 689ON Network GC (Agilent Technologies) system fitted with a 19091s-433 capillary column (Agilent Technologies) connected to an MS-5975 inert Mass Selective Detector (Agilent Technologies). The GC column was 30 metres in length with an internal diameter of 250µm and a film thickness of 0.25µm.

Following parameters described by Villas-Boas *et al.* (2003), the GC oven temperature was maintained at 60°C for 2 minutes before being ramped by 8°C a minute until the temperature reached 180°C. This temperature was held for 5 minutes before being ramped again at 40°C a minute until a temperature of 220°C was reached and held again for 5 minutes. Finally, the temperature was ramped by 40°C a minute until it reached 240°C. Helium was used as the carrier gas at a pressure of 3.45kPa.

The identities of mass spectrum peaks were derived from the MS-5975 database library. Confirmation by injection of known standards (maleic acid, oxalic acid, malonic acid, fumaric acid) was carried out prior to root exudate analysis

4.2.4 Statistical Validation of Data

Mycorrhizal colonisation data is presented as mean values of at least 3 replicates. Significant differences between treatments were tested by one way Analysis of Variance (ANOVA) using Fisher's LSD test. A 95% degree of confidence was utilised where the level of statistical significance was accepted at $p < 0.05$. Statistical analysis was carried out using the STATISTICA V7 (StatSoft Inc., 2005) statistics package (Zar, 1998).

4.3 Results

4.3.1 Visual Analysis of the Coal Substrate

C.dactylon growth was established in all treated columns and after 44 weeks, root penetration deep into the HC substrate had taken place (Figure 4.6). The highest degree of root proliferation was observed in the upper sections of the treated columns (Figure 4.8), with reduced proliferation down the length of the column.

Pronounced visual differences were observed between the untreated coal substrate and coal exposed to *C.dactylon* growth together with AMF and/or ECCN84 inocula. Substantial breakdown of coal had occurred in the top 0-40cm sections of the columns and showed degradation into the soil-like humic material that had been seen on the coal dumps investigated (Figure 4.7). Degradation in comparison to coal in HC control columns was also observed across treatments in the middle (Figure 4.9) and lower sections of columns (Figure 4.10).

Figure 4.6. A longitudinal view showing root penetration in a column containing *Cynodon dactylon* (right) compared to a hard coal control column (left) after 44 weeks incubation.

Figure 4.7. A close-up frontal view of the top 20cm section of a treated column planted with *Cynodon dactylon* showing breakdown of the coal substrate.

Figure 4.8. A close-up frontal view of the upper (0-40cm) section of a treated column in which the majority of root establishment took place.

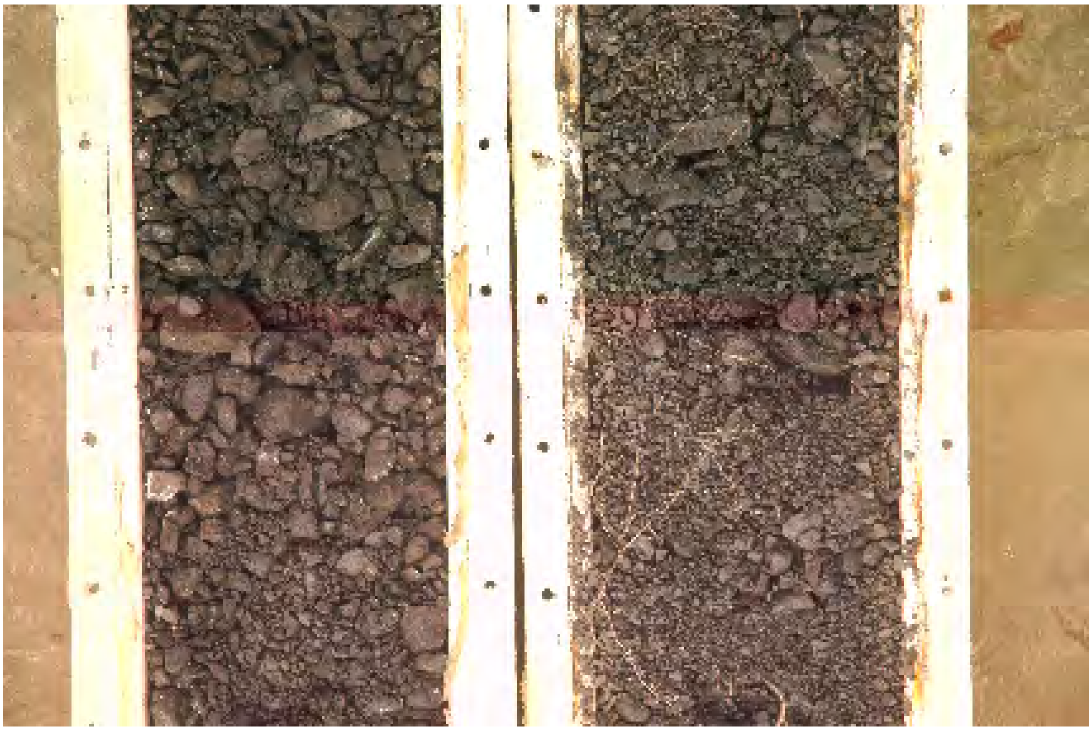

Figure 4.9. A close-up frontal view of the middle section of a treated column (right) compared to a hard coal control column (left). Increased coal degradation was also observed in the middle sections of treated columns.

Figure 4.10.A close-up frontal view of the bottom sections of a treated column (right) compared to a hard coal control column (left) showing increased coal degradation.

4.3.2 Mycorrhizal Colonisation of *Cynodon dactylon* Roots

Analysis of *C.dactylon* roots for mycorrhizal colonisation revealed that the majority of colonisation occurred in the upper layers of the column, with the highest percentages recorded from root samples harvested from the 20cm column depths (Table 4.1). Across all treatments, there was a significant reduction ($p < 0.05$) in mycorrhizal colonisation from the top to the bottom of the column.

The highest colonisation percentages at each point of analysis were recorded from the treatment HC + WC + *C.dactylon* + AMF + ECCN84. At the 20cm point of sampling, a colonisation percentage of 11.75% was recorded which was significantly higher than percentages recorded within the remaining 2 treatments of HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* (7.75% and 2% respectively). Similar trends between treatments were recorded at the 80cm and 140cm points of sampling with levels in the treatment HC + WC + *C.dactylon* + AMF + ECCN84 being significantly higher (Table 4.1). At the 20cm point of sampling, mycorrhizal colonisation in the treatment involving sole inoculation with AMF (HC + WC + *C.dactylon* + AMF) was significantly higher than in non-mycorrhizal (HC + WC + *C.dactylon*) roots, as may be expected. No mycorrhizal colonisation was recorded at the 140cm depth point of analysis in the HC + WC + *C.dactylon* treatment as well as the 80cm and 140cm depths in the treatment HC + WC + *C.dactylon* + AMF.

As in pot trials, the majority of the mycorrhizal colonisation observed in the roots of *C.dactylon* plants was of the *Paris*-type, although *Arum*-type colonisation features were also intermittently observed.

Table 4.1. Mycorrhizal colonisation percentages in the roots of *Cynodon dactylon* grown on hard coal amended with a weathered coal supplement at different levels within columns. Treatments involved un-inoculated control plants, plants inoculated with AMF and plants inoculated with both AMF and ECCN84.

Treatment	**Column Depth (cm)**	**Average AM fungal Root Colonisation Percentage**
HC + WC Supplement + *C.dactylon*	20cm	2.0%
HC + WC Supplement + *C.dactylon*	80cm	0.50%
HC + WC Supplement + *C.dactylon*	140cm	0
HC + WC Supplement + *C.dactylon* + AMF	20cm	7.75%
HC + WC Supplement + *C.dactylon* + AMF	80cm	0
HC + WC Supplement + *C.dactylon* + AMF	140cm	0
HC + WC Supplement + *C.dactylon* + AMF + ECCN84	20cm	11.75%
HC + WC Supplement + *C.dactylon* + AMF + ECCN84	80cm	1.50%
HC + WC Supplement + *C.dactylon* + AMF + ECCN84	140cm	0.25%

4.3.3 Scanning Electron Microscopy

SEM of coal particles harvested from the column study showed varying degrees of hyphal colonisation on the coal substrate surface. Hyphal colonisation on coal particles sampled from the column involving *C.dactylon* growth alone (HC + WC + *C.dactylon*) was very limited (Figure 4.11). Although hyphal colonisation was observed, this occurred as single strands. In comparison, an increase in surface hyphal colonisation of the coal substrate was observed on particles sampled from the treatment of HC + WC + *C.dactylon* + AMF with hyphal engulfment of smaller coal particles by numerous hyphal strands observed in this treatment (Figure 4.12).

The HC + WC + *C.dactylon* + AMF + ECCN84 treatment showed the highest hyphal colonisation of coal particles. Here, penetration of hyphae into the coal substrate was observed (Figure 4.13) with lines of shear fracture being associated with hyphal penetration. Figure 4.14 shows a cross sectional view of a shear fractured particle with extensive hyphal colonisation on the outside accompanied by hyphal penetration along the lines of fracture. Flaking of the coal substrate was also observed (arrow).

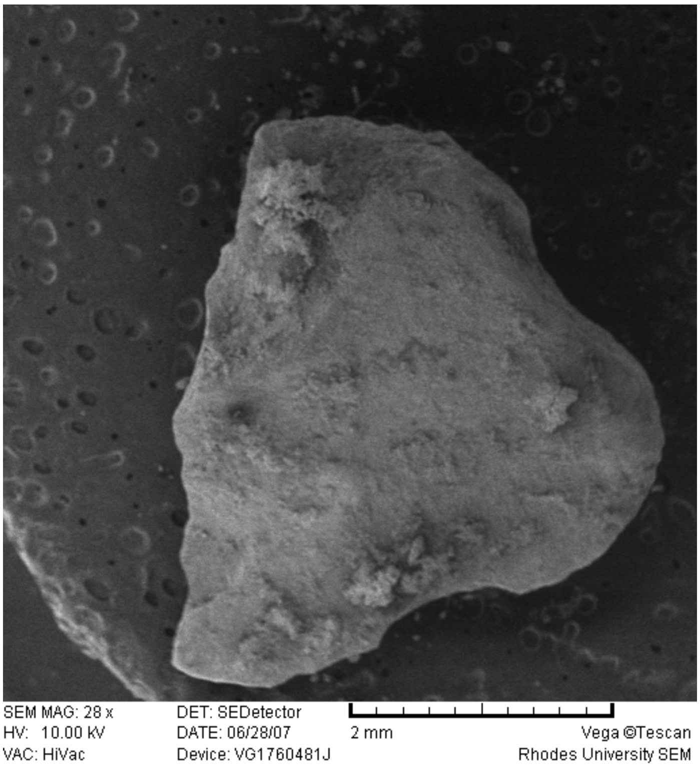

Figure 4.11. Scanning electron micrograph of coal particle harvested from a control column. Hyphal colonisation of coal substrate was very limited in the absence of arbuscular mycorrhizal fungal and EBRU Culture Collection Isolate Number 84 inocula**.**

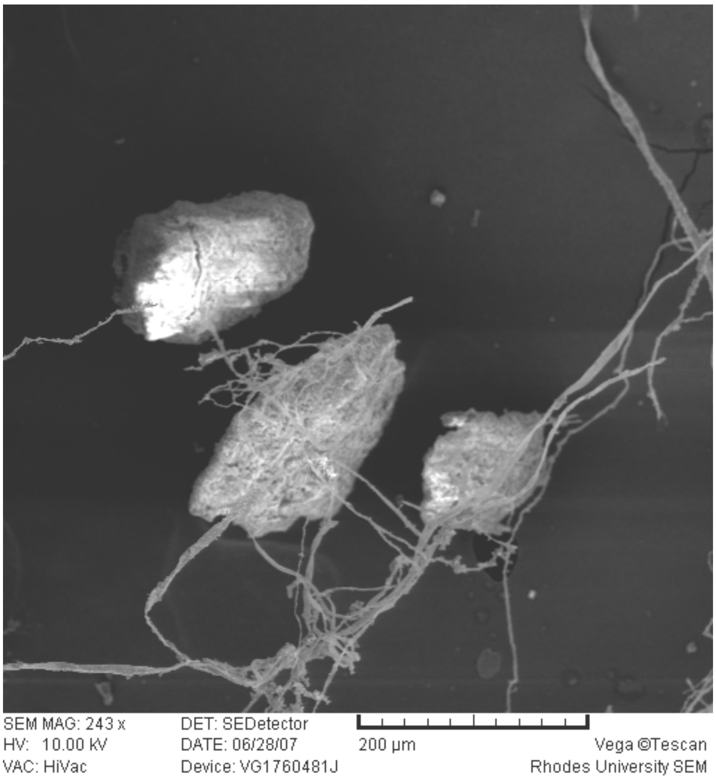

Figure 4. 12. Scanning electron micrograph showing hyphal engulfment of coal particles sampled from the hard coal column treated with *Cynodon dactylon* and arbuscular mycorrhizal fungi.

Figure 4.13. Scanning electron micrograph showing hyphal penetration of the surface of a coal particle sampled from a hard coal column treated with *Cynodon dactylon*, arbuscular mycorrhizal fungi and the EBRU Culture Collection Isolate Number 84. Coal surface shearing was observed along the line indicated by the arrow.

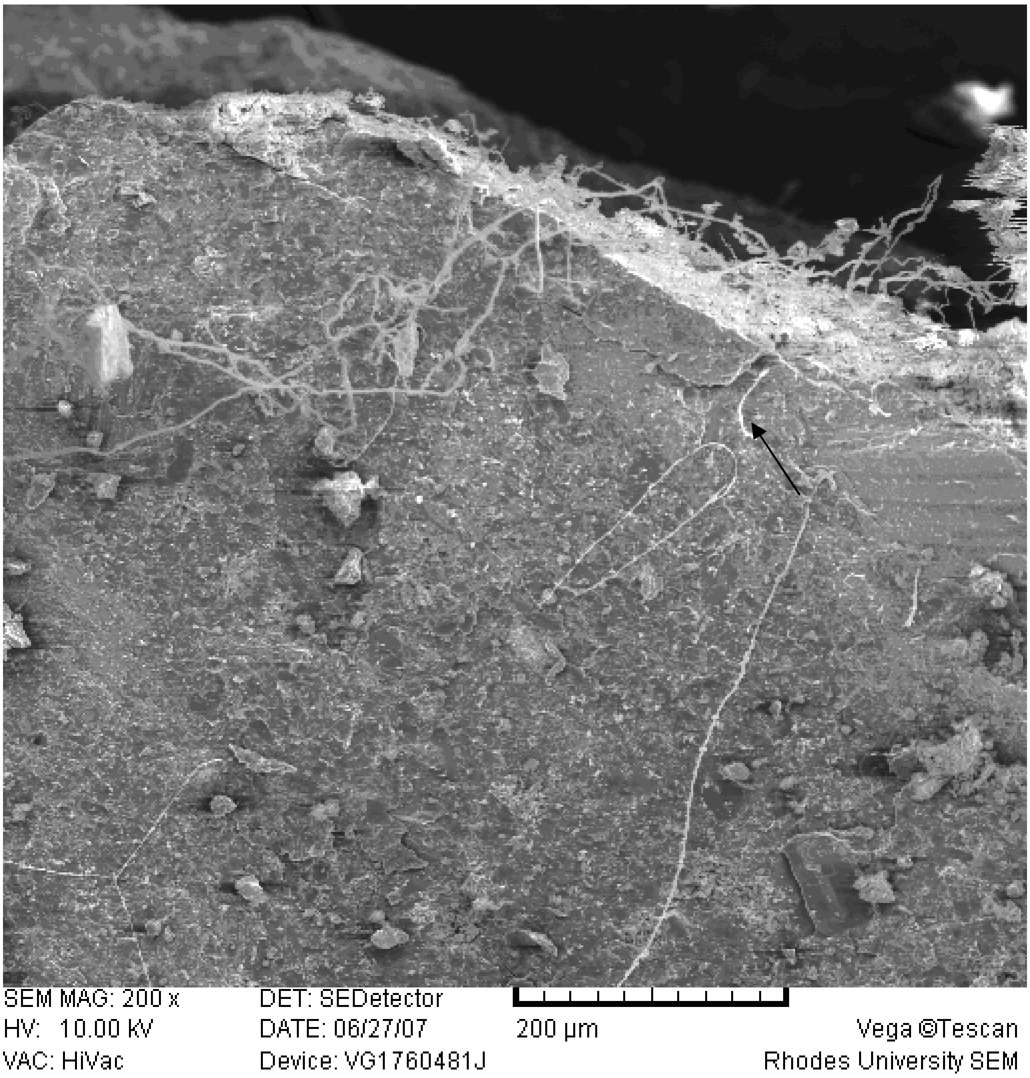

Figure 4.14. Scanning electron micrograph of a coal particle sampled from a hard coal column treated with *Cynodon dactylon*, arbuscular mycorrhizal fungi and the EBRU Culture Collection Isolate Number 84 showing extensive hyphal colonisation of the surface of the particle with hyphal penetration along the shear fracture line.

4.3.4 Size Fractionation of the Coal Substrate

Size fractionation analysis of coal packed into columns showed a reduction in larger size particles coinciding with an increase in smaller size particles when comparing treated coal to untreated control coal substrate (Figures 4.15, 4.16, 4.17 and 4.18).

Comparisons between the WC supplement utilised at trial initiation (T=0) and the same material from treated columns fractionated after 44 weeks (T=44) is shown in Figure 4.15. For the largest particle size (8mm), the highest mass was observed in the untreated WC supplement material at T=0 (28.14%). The 8mm particle size in all the treated columns after 44 weeks had been substantially reduced by 10.33%, 10.13% and 9.77% for the treatments HC + WC + *C.dactylon*, HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84 respectively. Reductions in mass in treated columns were not as pronounced for the 2mm particle size fraction with percentages in the treatments HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84 being lower but comparable to that measured in untreated WC supplement material. Here, the 2mm size fraction of the WC supplement material from the treatment HC + WC + *C.dactylon* (36.74%) was the same as the untreated cladding material (36.36%).

For the smaller size fractions (1mm, 500µm and >500µm), higher masses were measured in WC supplement material from treated columns in comparison to untreated WC supplement material at T=0. In treated columns, 1mm size fractions of 16.64%, 17.48% and 16.31% respectively were measured from the treatments HC + WC + *C.dactylon*, HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84 in comparison to 9.52% measured in untreated WC supplement material. For the 500µm size fraction, masses of 13.17%, 15.2% and 16.31% were measured from the treatments HC + WC + *C.dactylon*, HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84 respectively in comparison to 10.39% measured in untreated WC supplement material. Finally, for the smallest size fraction analysed (>500µm), increases in mass in treated WC supplement material in comparison to untreated WC supplement material were also observed. Here, a mass of 15.58% was recorded from the untreated material. For WC supplement material that underwent treatment with HC + WC + *C.dactylon,* HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* + AMF + ECCN84, higher masses of 23.13%, 24.19% and 24.5% respectively were recorded.

Size fractionation analysis of the area directly below the top WC supplement layer (20-40cm), the middle of each column (70-90cm) and the bottom of each column (120-140cm) showed similar trends (Figure 4.16, Figure 4.17, Figure 4.18). Analysis of the 20-40cm sections of each column revealed lower masses for the 8mm and 2mm particle size fractions and higher masses for the 1mm, 500µm and >500µm particle size fractions in treated HC in comparison to untreated HC material at T=0 and T=44 (Figure 4.16). For the smaller particle size fractions (1mm, 500µm and >500µm) the highest mass was recorded from the treatment HC + WC + *C.dactylon* + AMF + ECCN84. Reductions in larger particle size fractions and increases in smaller size fractions in HC substrate sampled from treated columns at the 70-90cm and 120-

140cm sections of columns in comparison to untreated material were also observed (Figure 4.17, Figure 4.18). Differences between treated and untreated HC coal substrates were not as enhanced in the middle (70-90cm) and especially the bottom (120-140cm) sections of the columns. Slight alteration of untreated HC substrate did take place from T=0 to T=44 with reductions of larger size particles and increases in smaller size particles also observed.

As single columns were destructively harvested to obtain size fractionation results, statistical analysis between treatments was not possible.

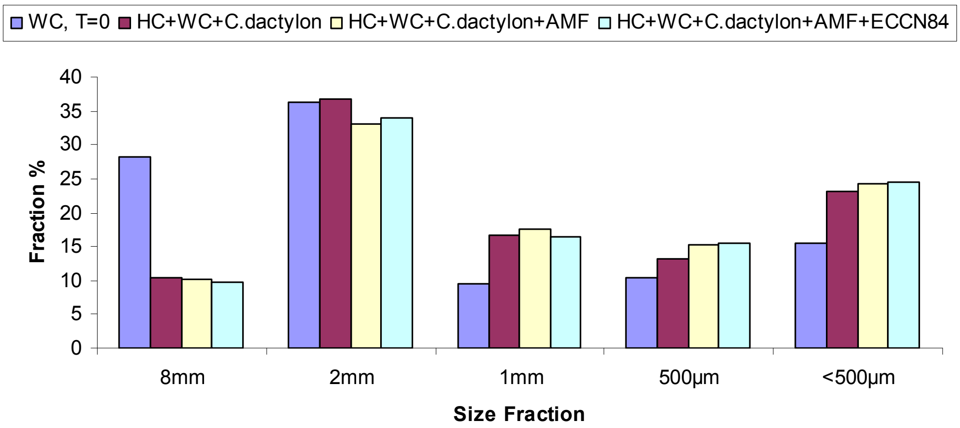

Figure 4.15. Size fraction percentage of weathered coal supplement material in the top 20cm of columns that underwent various treatments over 44 weeks. The weathered coal supplement material at T=0 is compared to material exposed to *Cynodon dactylon*, *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi and *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84 at T=44.

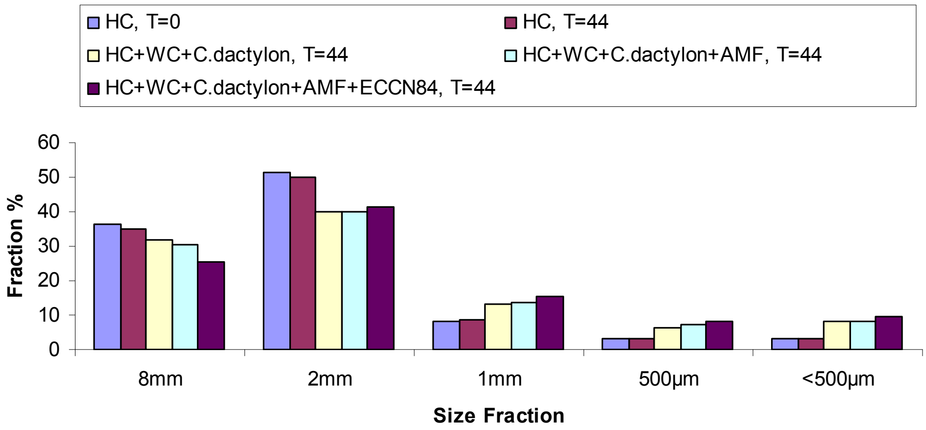

Figure 4.16. Size fraction percentage of coal substrate harvested from the 20-40cm sections of control and treated columns after 44 weeks. Hard coal material at T=0 is compared to material exposed to *Cynodon dactylon*, *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi and *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84 at T=44.

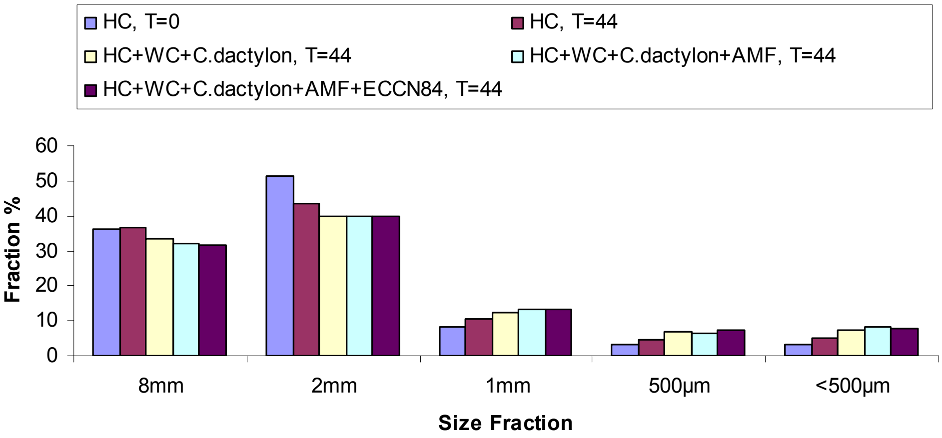

Figure 4. 174. Size fraction percentage of coal substrate harvested from the 70-90cm section of control and treated columns after 44 weeks. Hard coal material at T=0 is compared to material exposed to *Cynodon dactylon*, *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi and *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84 at T=44.

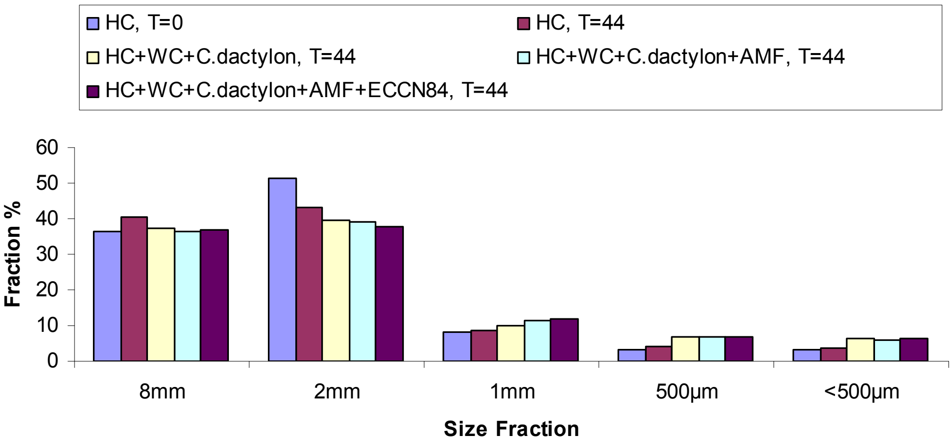

Figure 4.18. Size fraction percentage of coal substrate harvested from the 120-140cm section of control and treated columns after 44 weeks. Hard coal material at T=0 is compared to material exposed to *Cynodon dactylon*, *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi and *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Number 84 at T=44.

4.3.5 Root Exudate Analysis for Organic Acids

GC-MS root exudate analysis of non-mycorrhizal (Figure 4.19, Table 4.2) and mycorrhizal (Figure 4.20, Table 4.3) plants revealed the presence of a variety of compounds possibly exuded from the plants or present within the rhizosphere. Chlorinated and aromatic compounds were identified in both non-mycorrhizal and mycorrhizal colonised root exudates evaluated. Organic acids were not identified within exudates of non-mycorrhizal plants, however the molecule 2-Butenedioic acid (E)- dimethyl ester was present within the root exudates of mycorrhizal plants (denoted by asterisk, Figure 4.20), this a methylated form of fumaric acid.

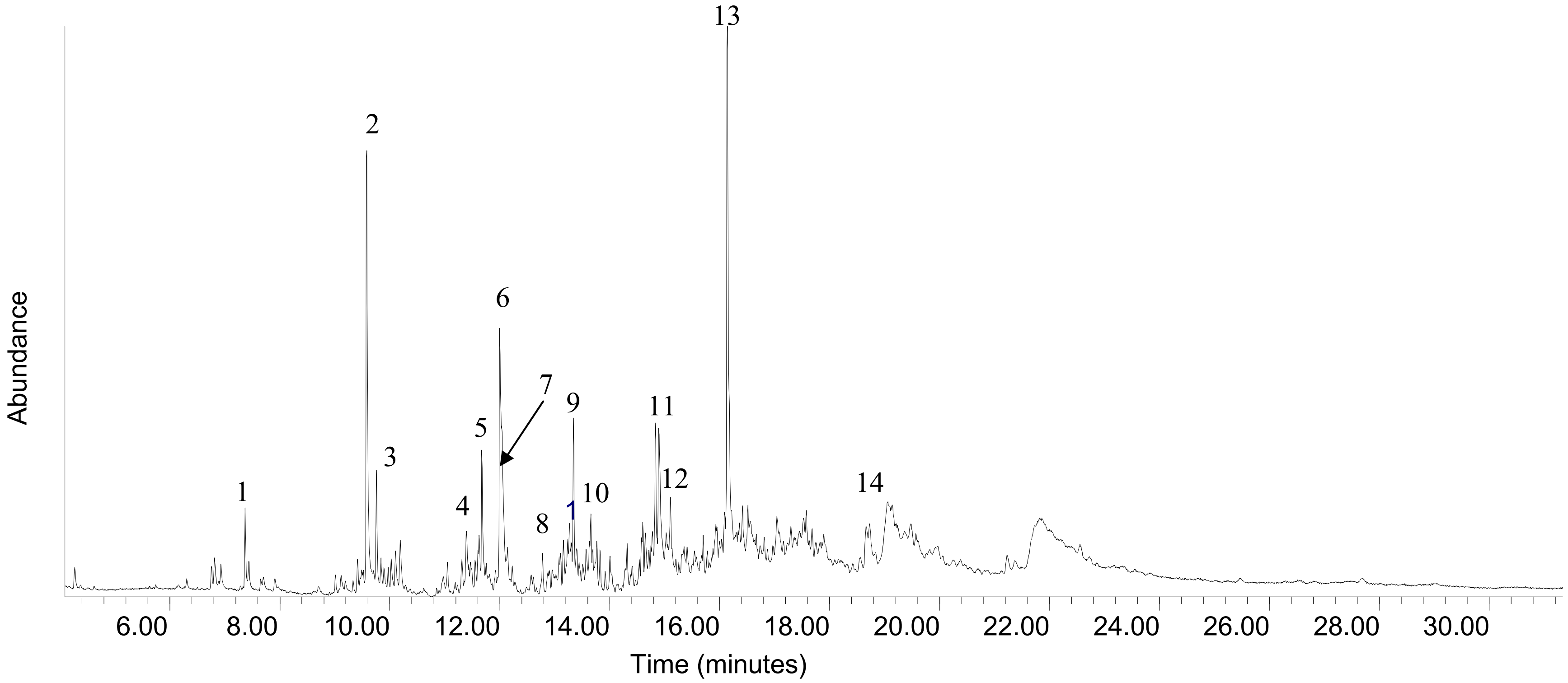

Figure 4.5. Gas Chromatography–Mass Spectroscopy chromatogram showing compound peaks detected at various retention times obtained from analysis of root exudates collected from non-mycorrhizal plants. Identity of compounds based on the Mass Spectroscopy library are listed in Table 4.2.

Table 4.2. Retention times and putative identity of compounds present within root exudates collected from non-mycorrhizal plants. Times relate to compound peaks detected from Gas Chromatography-Mass Spectroscopy analysis and compound identities were derived from the Mass Spectroscopy database library (Figure 4.19).

Peak Number	Retention Time (Minutes)	Compound
1	7.366	Decane, 3,6-dimethyl-
2	9.578	Benzene, 1,3-bis(1,1-dimethylethyl)
3	9.758	Dodecane
4	11.391	Heptadecane, 2,6,10,15-tetramethyl
5	11.676	Silane, trichlorooctadecyl-
6	12.000	Phenol, 2,4-bis(1,1-dimethylethyl)
7	13.342	Heptadecane, 9-octyl-
8	13.656	Heptadecane, 9-octyl-
9	14.603	2-Bromo dodecane
10	14.834	Heneicosane
11	14.894	Hexadecanoic acid, methyl ester
12	15.104	Triacontane
13	16.137	Octadecanoic acid, methyl ester
14	19.060	1-Dimethyl(trimethylsilylmethyl)silyloxy-3-phenylprop-2-ene

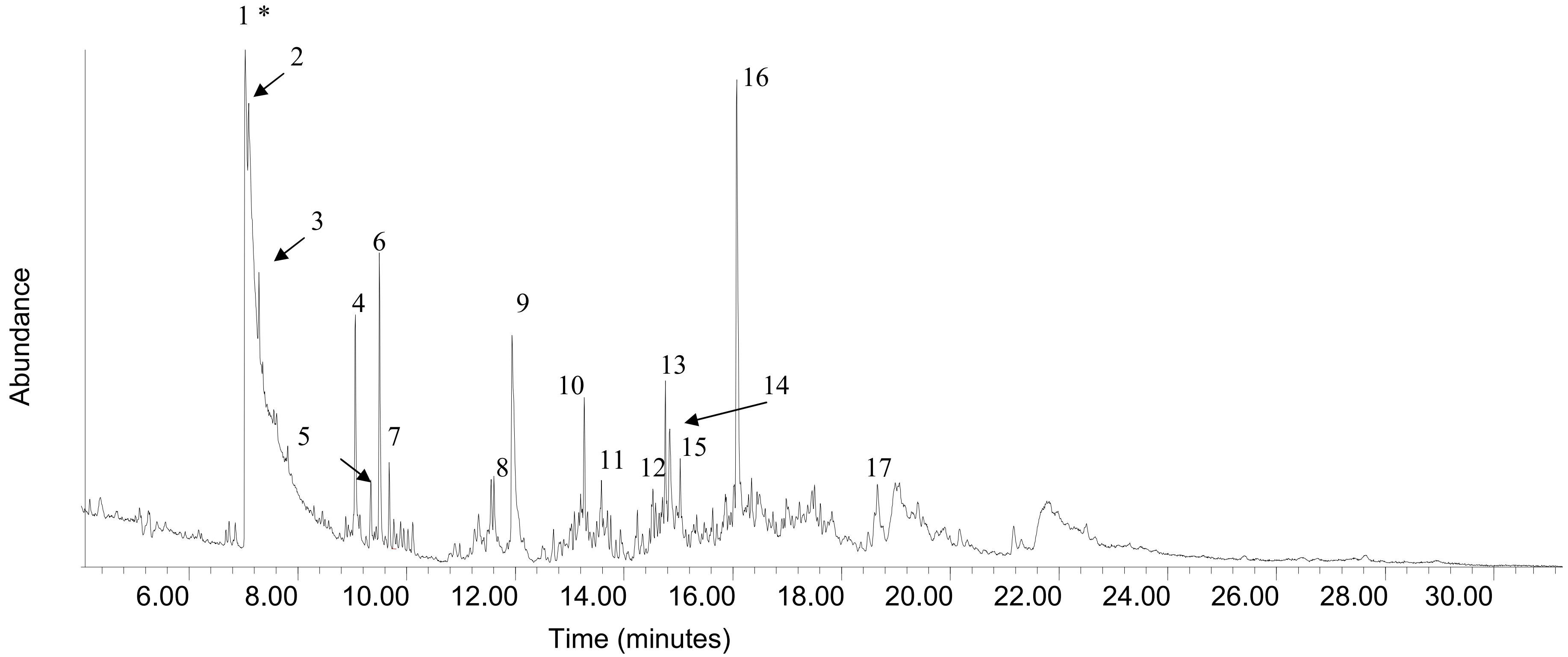

Figure 4.206. Gas Chromatography-Mass Spectroscopy chromatogram showing compound peaks detected at various retention times obtained from analysis of root exudates collected from mycorrhizal plants. Identity of compounds based on the Mass Spectroscopy library are listed in Table 4.3.

Table 4.3 Retention times and putative identity of compounds present within root exudates collected from mycorrhizal plants. Times relate to compound peaks detected from Gas Chromatography-Mass Spectroscopy analysis and compound identities were derived from the Mass Spectroscopy database library (Figure 4.20)

Peak Number	Retention Time (Minutes)	Compound
1	7.108	2-Butenedioic acid (E)-, dimethyl ester
2	7.170	Thiazole, 4,5-dimethyl-
3	7.360	Decane, 3,7-dimethyl –
4	9.131	Benzene
5	9.417	Tridecane, 6-propyl-
6	9.575	Benzene, 1,3-bis(1,1-dimethyl)-
7	9.755	Tridecane, 6-propyl
8	11.68	Heneicosane
9	12.014	Phenol, 2,4-bis(1,1-dimethyl)
10	13.42	Heneicosane
11	13.654	Hexatriacontaine
12	14.605	Tritetracontaine
13	14.834	Triacontaine
14	14.911	Hexadecanoic acid, methyl ester
15	15.108	Tetrapentacontaine
16	16.145	Octadecanoic acid, methyl ester
17	19.059	d-Ribose, 2-deoxy-bis(thiononyl)-dithioacetal

4.4 Discussion

4.4.1. Visual Analysis of the Coal Substrate

Degradation of the coal substrate was observed in treated columns which appeared to simulate the generation of a humic soil-like material observed in the Navigation Mine discard coal dumps. The ability of plants to weather mineral matter has been described (Hinsinger *et al*., 2001; Puente *et al*., 2004) and AMF rhizospheric fungi have been implicated in these processes (van Breemen *et al*., 2000a; van Breemen *et al*., 2000b). The absence of this effect in the untreated control columns and an increased effect in columns inoculated with AMF and ECCN84 indicates that both organisms may have played a role in the generation of this result.

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4.4.2 Mycorrhizal Colonisation of *Cynodon dactylon* Roots

Mycorrhizal colonisation was observed in the roots of *C.dactylon* plants grown in the column study although this was generally lower than colonisation in pot trials (Table 4.1). As in the pot trials, *Paris*-type colonisation predominated. Rates and patterns of mycorrhizal colonisation have been shown to vary with mycorrhizal species as well as often being determined by the plant host and rhizospheric environment (Pelletier and Dionne, 2004).

The mycorrhizal colonisation recorded in treatments not inoculated with AMF (Table 4.1) demonstrated that background AMF colonisation occurred. However, as may be expected, mycorrhizal colonisation increased for treatments where mycorrhizal inoculation was applied. The addition of ECCN84 together with AMF (HC + WC + *C.dactylon* + AMF + ECCN84) increased the mycorrhizal colonisation in comparison to the treatment involving sole inoculation with AMF (HC + WC + *C.dactylon* + AMF). The similar observation measured in pot trials strengthened the suggestion that ECCN84 coal degradation activity and the supply of nutrients to the plant is may be driven by the release of organic C. An additional AMF population may be required to mediate this effect.

Across all treatments, the highest mycorrhizal colonisation percentages in plant roots were recorded in the top 20cm sections of each column where the greatest breakdown of coal particle size was seen. This further suggested that a relationship may exist between *C.dactylon* root growth, AMF colonisation, the presence of ECCN84 and coal biodegradation.

4.4.3 Scanning Electron Microscopy

SEM of coal particles from treated columns revealed an increase in surface hyphal colonisation on particles exposed to *C.dactylon* growth together with inoculation of AMF and AMF/ECCN84. Shear fracturing of coal particles was also observed in inoculated columns which was absent in the untreated controls. Hyphal penetration into fractures was also seen (Figure 4.13). The initial concept of "rock eating mycorrhizal fungi" was first described by Jongmans *et al*. (1997) who observed similar hyphal penetration of a number of minerals from European coniferous forests. Similar observations were also made by van Breemen *et al*. (2000a) who visualised mycorrhizal hyphal penetration of feldspar as well as grooves formed by hyphae on the mineral surface. The observation of hyphal colonisation and penetration of coal particles in the column study suggested that fungal inocula were involved in the coal degradation phenomenon. Differentiation between AM and ECCN84 hyphal strands, however, was not possible.

4.4.4 Size Fractionation of the Coal Substrate

The size fractionation analysis of the coal column packing material seemed to quantify the degradation of coal substrate that was apparent in the manual observation of the system. Analysis of the WC supplemented treatments (Figure 4.15) showed clear particle size reduction when comparing untreated material at the start of the trial (T = 0) and material that underwent various treatments for 44 weeks. At T = 0, WC supplement material alone mainly consisted of larger coal particles (8mm and 2mm coal particles) with these particles making up over 60% percent of the cladding material. Coal particles sized 1mm and below were less abundant. However, after 44 weeks of treatment, a shift in particle size was observed with percentages of smaller particle sizes increasing and percentages of particle sizes decreasing. This observation was made across all treatments further demonstrating a clear degradation of coal related to the treatments applied. Where coal degradation did occur in the presence of

C.dactylon alone, this was increased in treatments involving the addition of AMF and/or ECCN84 inocula. Size fractionation of the coal substrate in the lower section of the columns also showed increased degradation in treated columns compared to untreated columns.

The potential role of fungal growth in coal degradation in the *C.dactylon*/coal system was further supported by observations of mycorrhizal colonisation for the various treatments and SEM visualisation of the possible process.

4.4.5 Root Exudate Analysis for Organic Acids

Analysis of root exudates collected from non-mycorrhizal plants revealed the presence of a variety of compounds such as hydrocarbons, chlorinated compounds, aromatic compounds and fatty acids (Table 4.2). However, root exudates from plant inoculated with AMF (Table 4.3) also showed the presence of 2-Butenedioic acid (E) dimethyl ester, a methylated form of fumaric acid. Methylation of organic acids present within root exudates would have occurred during sample derivitisation. Jongmans *et al*. (1997) had suggested that the penetration by mycorrhizal hyphae of Al silicate minerals was mainly aided by organic acid exudates. Organic acids are able to enhance the dissolution of primary minerals thereby making essential nutrients available for plant growth (Landeweert *et al*., 2001).

Studies, primarily on ectomycorrhizal fungi, have revealed that like many other fungi, mycorrhizal fungi are able to exude low molecular weight organic acids from their hyphal tips (Lapeyrie *et al*., 1987). The role of oxalic acid in mineral weathering has been reported in particular (Gadd, 1999; Landeweert *et al*., 2001), however, citric, formic, acetic, malic, succinic, malonic, maleic, lactic, aconitic and fumaric acids have also all been identified as playing a role in mineral weathering (van Breemen *et al*., 2000b; van Hees *et al*., 2000). The production of fumaric acid by mycorrhizal fungi has been associated with P solubilisation (Ouahmane *et al*., 2007). Plants themselves do produce a wide range of organic acids that can activate and fix potential nutrients as well as chelate such elements as Al (Wang and Zhou, 2006).

4.5 Conclusions

- The development of the column reactor system enabled a simulation of the coal dump environment and it was possible to replicate the breakdown of HC to humic soil-like substrate in this system in the presence of *C.dactylon* growth as well as AMF and ECCN84 inoculum.

- The visual observations of coal degradation were confirmed in measurement of particle size reduction in size fractionation studies.

- The breakdown of the coal substrate was further confirmed in SEM studies which showed hyphal penetration of the coal substrate comparable to previous descriptions of "rock-eating fungi" which contributed to soil formation. Fracturing of the coal substrate with chipping/sloughing into smaller pieces was simultaneously observed.

- The breakdown of the coal substrate was linked to the production of fumaric acid in AMF inoculated systems in comparison to un-inoculated controls. This observation also relates to previous reports of organic acid exudation by fungi in the breakdown of minerals.

CHAPTER 5:

SIMULATION OF THE BITUMINOUS HARD COAL DISCARD DUMP ENVIRONMENT 2: CHEMICAL CHANGES

5.1 Introduction

Initial studies had shown that in the presence of *C.dactylon* and associated mycorrhizal and non-mycorrhizal rhizospheric fungi, the biodegradation of coal into a humic soil-like material takes place. This was confirmed in column reactor studies set up to simulate the coal dump environment and structural changes were reported in Chapter 4. Chemical changes that occurred within the column reactors were also monitored during the course of the above investigation and reported here.

5.2 Methods

5.2.1 Sampling

Chemical analysis of plant and coal substrate material was undertaken as previously described (Section 4.2.2). Here however, ~15g samples of coal were removed at 20cm intervals down the length of the column, with the first sampling point being 20cm into each column. In reiteration, sampling thus consisted of triplicate samples from 2 separate columns.

5.2.2 Analysis

5.2.2.1 Humic Acid

To measure water soluble humic acid fractions, 10g of coal sample from each sampling point was diluted in 100ml of distilled water. Triplicate 7ml samples from this solution were acidified to below pH 1 by the addition of 150µl concentrated hydrochloric acid (Merck, 306 20 LP) to precipitate the humic acid fraction. Samples were incubated at room temperature for 24 hours before centrifugation in an Eppendorf 5810 R centrifuge at 4000rpm for 90 minutes at 10°C. The supernatant was removed and the pellet re-suspended in 7ml 0.1M sodium hydroxide (Analytical Reagents, RPCK3320) (Magi *et al.*, 1995).

Into each sample, 1.05ml of 200gL^{-1} sodium carbonate (Merck, 1.06392.0500) was added and the sample agitated briefly. A 210µl aliquot of each sample was placed into a well of a microtitre plate and 70µl of Folin-Ciocalteus phenol reagent (Merck, 1.09001.0100) was added to each sample. Samples were incubated at room temperature for 60 minutes. The OD of each sample was then measured at 750nm using a Biotek Instruments PowerWave microtitre plate reader.

Standard humic acid solutions of 0, 20, 40, 60, 80, 100, 200 and 300ppm were prepared by diluting the appropriate amount of humic acid (Fluka, 53680) in 0.1M sodium hydroxide and analysed as above. The humic acid concentrations in each sample were read from a standard curve ($y = 0.003x$, $R^2 = 0.9312$) (Appendix 4). Humic acid production within the columns provided an indication of coal breakdown.

5.2.2.2 Total Organic Carbon

10ml of the sample solution prepared for humic acid analysis was used for Total Organic Carbon (TOC) measurements. Liquid samples were analysed using the Teledyne Tekmar Apollo 9000 Combustion Total Organic Carbon analyser. Potassium hydrogen phthalate (Merck, 1.04874.0250) of 100ppm, 200ppm and 400ppm concentrations was used to calibrate the Analyser as per manufacturer's instructions (Appendix 4).

Samples were centrifuged at 80000rpm for 5 minutes at 10°C in an Eppendorf 5810 R centrifuge to pellet any excess particulate material and the supernatant collected. Samples were analysed by direct injection into the analyser.

5.2.2.3 Moisture

Approximately 1g of coal sampled from each analysis point was pre-weighed to obtain a wet weight and then dried for 48h at 60°C in a Labcon Incubator. Samples were then re-weighed to obtain the dry weight from which the percentage moisture content was calculated (Okalebo *et al*., 1993). Moisture content analysis down the column was analysed to provide an indication of water percolation down into the columns that might contribute to acid-mine drainage (Davis *et al*., 1999).

5.2.2.4 Oxygen

Oxygen levels down the column length was determined using an Eijkelkamp P1.66-1 Oxygen Diffusion Meter. The steel probe of the meter, approximately 1 metre long, was pressed down into the coal substrate from the top of the column and oxygen readings taken 20, 40 and 60cm into the column. The averages of readings at these three points of analysis were combined and utilised. Oxygen levels within columns were analysed to provide an indication of oxygen ingress into columns (discard HC dumps) which could contribute to spontaneous combustion and acid-mine drainage (Carpentier *et al*., 2005).

5.2.2.5 pH

Further 30ml aliquots of solutions prepared for humic acid analysis were briefly agitated before pH was measured a WTW 330 pH meter. pH levels within columns provided an indication of possible acid-mine drainage formation.

5.2.2.6 Elemental Analysis of Coal and Soil

Triplicate 30g samples of HC, WC and soil utilised in pot and column trials were dried at 60°C overnight and sieved (45µm). Samples were analysed by The

Agricultural Research Council (ARC) Institute of Soil, Climate and Water (Pretoria, South Africa) as well as Mintek Laboratories (Randburg, South Africa) using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), an analytical method capable of quantifying a wide range of elements from trace to ultra-trace levels (Kulkarni *et al.*, 2007).

5.2.2.7 Elemental Analysis of Plant Material

Elemental analysis of plant material was conducted using Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES).

Acid digestion of plant material prior to ICP-OES analysis was based on the United States Environmental Protection Agency (EPA) method 3050 B. Approximately 5g samples of *C.dactylon* roots (sampled from the 20cm point of analysis) and shoots were harvested from each treated column and dried at 60°C for 48 hours in a Labcon Incubator. Dried samples were crushed using a pestle and mortar and 1g of crushed material placed in a 100ml conical flask to which 10ml 50% nitric acid (Merck, 446 50 40 LC) was added. The opening of the conical flask was covered with a watch glass and the sample was refluxed for 10 minutes at 95°C on a Labinco L-81 Hotplate Stirrer. Five ml concentrated nitric acid was added to the sample and the sample refluxed at 95°C for a further 30 minutes. Two ml distilled water and 3ml 33% hydrogen peroxide (Merck, 306 38 00 LP) was then added to the sample. Addition of 33% hydrogen peroxide to the sample produced effervescence (due to carbon oxidation) and 33% hydrogen peroxide was continually added until a total volume of 10ml had been added and effervescence annulled. Samples were then refluxed for 1 hour at 95°C before 10ml hydrochloric acid (Merck, 306 20 LP) was added to the samples. Samples were diluted to a total volume of 100ml and stored at 4°C. All glassware utilised in sample preparation was soaked in 50% nitric acid for 24 hours prior to use.

Prepared acid-digested plant samples were filter-sterilised through a 0.45µm nylon membrane (PALL Life Sciences, 6607) prior to ICP-OES analysis using a Thermo ICAP-6300 spectrometer. ICP spectrometry is a sensitive elemental analysis technique that can detect a wide range of elements at low concentrations and has previously been used in multi-element analysis of acid-digested plant material (Zarcinas *et al*, 1987).

5.2.3 Statistical Validation of Data

All data is presented as mean values of at least 3 replicate readings with standard errors reported. TOC, humic acid, moisture and pH standard errors at each point of analysis are listed in Appendix 4. Significant differences between treatments were tested by one way Analysis of Variance (ANOVA) using Fisher's LSD test. A 95% degree of confidence was utilised where the level of statistical significance was accepted at $p < 0.05$. Statistical analysis was carried out using the STATISTICA V7 (StatSoft Inc., 2005) statistics package (Zar, 1998).

Figures depicting results obtained from column analysis are presented as depth profile graphs with the Y axis representing the height of the column in cm, thus being representative of the point of the column at which analysis took place (Figure 5.1).

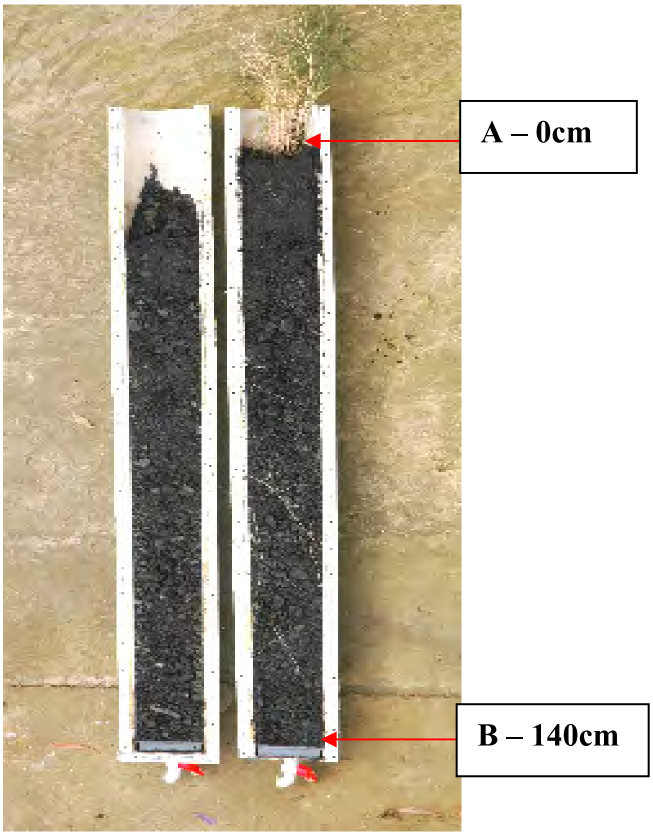

Figure 5.1. Vertical profile of a hard coal control and treated column. In results, the top of the column (A) is referred to as point 0cm and the bottom of the column (B) as point 140cm.

5.3 Results

5.3.1 Humic Acid

Humic acid analysis within the columns, as may be expected, showed higher levels of humic acid were generated from HC supplemented with WC and exposed to *C.dactylon* growth in a variety of treatments in comparison to untreated HC (Figure 5.2).

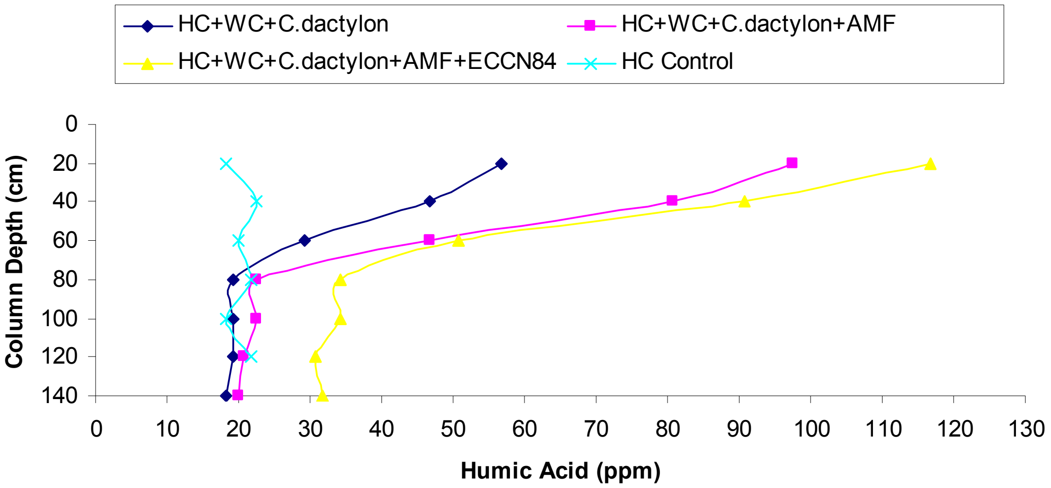

Figure 5.2. Depth profile graph illustrating humic acid levels at various depths within treated and control columns. Treatments compared; Hard Coal + Weathered Coal Supplement + *Cynodon dactylon*, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control.

At the 20cm depth where initial readings were taken, humic acid levels were highest in the treatment HC + WC + *C.dactylon* + AMF + ECCN84 (116.67ppm) followed by levels in the treatments consisting of HC + WC + *C.dactylon* + AMF (97.50ppm) and HC + WC + *C.dactylon* (56.67ppm) respectively. Humic acid levels in these treatments were all significantly higher ($p < 0.05$) than the humic acid level in the HC column (18.17ppm) at this point. Humic acid levels within the treatment of HC + WC + *C.dactylon* + AMF + ECCN84 were also significantly higher than levels within the treatment lacking inoculation with either AMF or AMF and ECCN84 (HC + WC + *C.dactylon*). Humic acid levels within this treatment were 51.43% and 16.43% higher than levels within the treatments HC + WC + *C.dactylon* and HC + WC + *C.dactylon* + AMF respectively.

Humic acid levels across all treatments gradually reduced down the lengths of each treated column. However, levels within the treatment HC + WC + *C.dactylon* + AMF + ECCN84 remained highest. From the 80cm mark to the base of each column (120cm), humic acid levels in the treated columns though generally higher were not significantly different to the HC control. Humic acid levels in HC control columns remained constant at approximately 20ppm down the length of the column.

5.3.2 Total Organic Carbon

Concentrations of available water soluble C within the column rhizospheres were determined by TOC analysis. TOC levels within treated columns remained higher than levels within un-treated HC control columns down the lengths of columns (Figure 5.3)

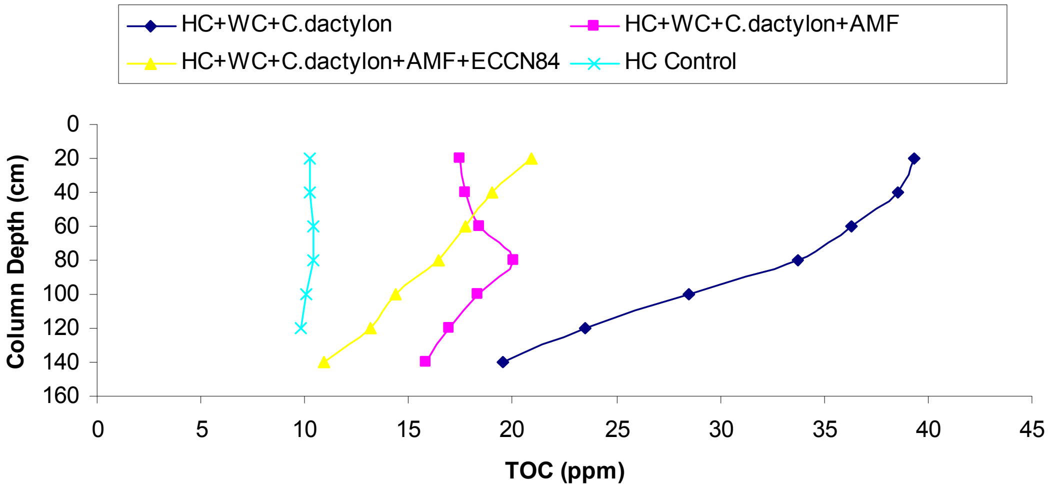

Figure 5.3. Depth profile graph illustrating total organic carbon levels at various depths within treated and control columns. Treatments compared; Hard Coal + Weathered Coal Supplement + *Cynodon dactylon*, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control.

In contrast to humic acid results, the highest TOC was observed in the treatment HC + WC + *C.dactylon.* At the top of the column (20cm), the TOC concentration in this column was 39.35ppm which was significantly higher ($p < 0.05$) than the TOC concentration in the hard coal control column (10.21ppm) as well as levels within the two remaining treatments of HC + WC + *C.dactylon* + AMF and HC + WC +

C.dactylon + AMF + ECCN84 (17.44ppm and 20.89ppm respectively). This represented TOC concentration increases of 55.68% and 46.92%. As observed in humic acid analysis, TOC concentrations gradually reduced down the length of each treated column. However, even with this reduction, TOC levels at each point of analysis within the treatment HC + WC + *C.dactylon* were significantly higher than in the HC control column, this was also the case for levels within the treatment HC + WC + *C.dactylon* + AMF. TOC levels within the treatment HC + WC + *C.dactylon* + AMF + ECCN84 were significantly higher than levels within the HC control above the 100cm point of analysis.

TOC concentrations in the HC control column remained at ~10ppm down the length of the column.

5.3.3 Moisture

Apart from the top 0-20cm sections of each column, moisture content percentages across treated columns were lower than percentages found in the untreated HC control column (Figure 5.4).

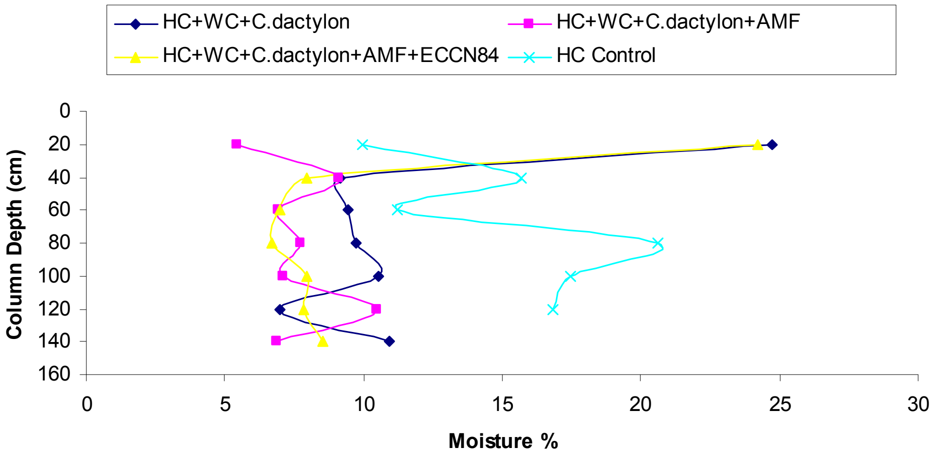

Figure 5.4. Depth profile graph illustrating moisture content percentages at various depths within treated and control columns. Treatments compared; Hard Coal + Weathered Coal Supplement + *Cynodon dactylon*, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control

At the 20cm depth, the treatments of HC + WC +*C.dactylon* and HC + WC + *C.dactylon* + AMF + ECCN84 had higher moisture content percentages of 24.73%

and 24.21% respectively in comparison to the HC control column which had a moisture content percentage of 9.97%. Both these results were significantly higher ($p < 0.05$) than that of the HC control, but not significantly different to each other. However, at this point of analysis, for the remaining treatment (HC + WC + *C.dactylon* + AMF), the moisture content percentage (5.42%) was lower than that obtained in the HC control column, though this was not a significant difference.

Below the 20cm point of analysis, moisture content percentages between treatments were relatively similar remaining between ~7% and 10%, lower than levels within the HC control.

5.3.4 Oxygen

A decrease in oxygen percentage was observed in both HC control and treated columns (Figure 5.5). The greatest reduction in oxygen, in comparison to the oxygen percentage in air (21%), was measured in the treatment of HC + WC + *C.dactylon* + AMF + ECCN84 (19.8%). Oxygen percentages of 20.7%, 20.7% and 20.4%, were measured in the HC control, the treatment HC + WC + *C.dactylon* and the treatment of HC + WC + *C.dactylon* + AMF respectively. The differences observed were not statistically significant.

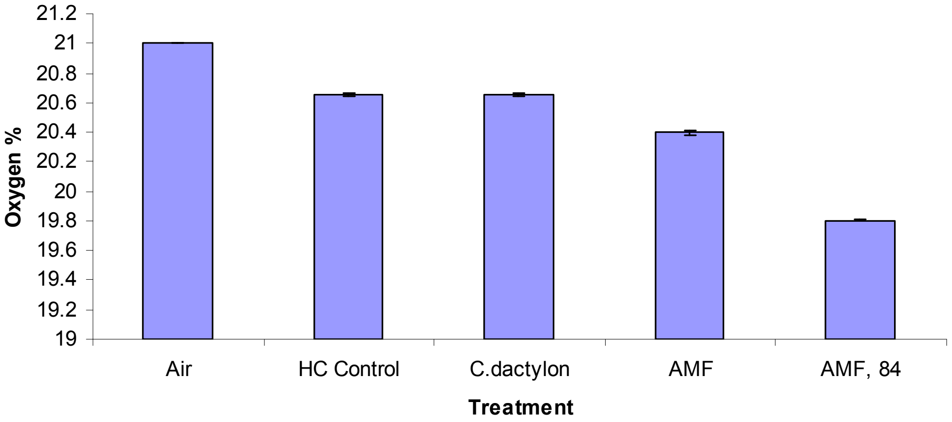

Figure 5.5. Oxygen percentages within control and treated columns in comparison to the oxygen percentage in air (21%). The average percentage of oxygen measurements from three points (20, 40 and 60cm) within each column is represented in the graph. Treatments compared; Hard Coal Control, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon*, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84.

5.3.5 pH

pH levels in columns remained between pH 3.6 and 4.5 across all treatments (Figure 5.6) and were more alkaline in comparison to pH levels within the HC control that remained at approximately pH 3.5 down the column length. At the 20cm point of analysis, the highest pH levels were recorded in the treatment HC + WC + *C.dactylon* + AMF + ECCN84 (pH 4.50) which was significantly higher ($p < 0.05$) than levels within the HC control column at the same mark (pH 3.64). pH levels in the remaining treatments of HC + WC + *C.dactylon* + AMF and HC + WC + *C.dactylon* were also significantly higher (pH 4.12 and pH 4.10 respectively). pH levels in treated columns gradually decreased down the lengths of columns until below the 80cm mark in each column, pH levels were generally not significantly higher than levels in the HC control column. However, at the 120cm point of analysis, the pH level in the treatment HC + WC + *C.dactylon* was significantly higher in comparison to the HC control.

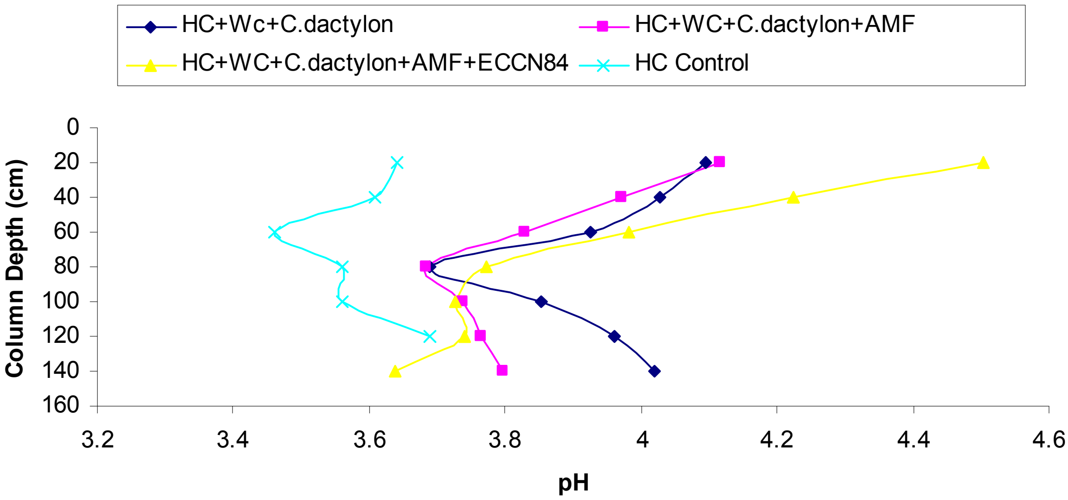

Figure 5.6. Depth profile graph illustrating pH levels at various depths within treated and control columns. Treatments compared; Hard Coal + Weathered Coal Supplement + *Cynodon dactylon*, Hard Coal + Weathered Coal Supplement + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control

5.3.6 Elemental Analysis of Coal and Soil

Elemental composition of the HC, WC and soil utilised in the column study were determined in order to measure the concentrations of various elements, mainly heavy metals, to which *C.dactylon* plants would be exposed. The results are reported Table 5.1, Table 5.2 and Table 5.3.

ICP-MS analysis revealed that concentrations of Mn, Zn and Pb were negligible within the HC, WC and soil utilised in trials (Table 5.1). For each metal, percentage concentrations of <0.00005ppm were obtained in each substrate. Al percentages were relatively similar in all 3 substrates with concentrations of 0.061ppm, 7.9ppm and 8.7ppm recoded in the HC, WC and soil respectively. Fe concentrations in the HC and soil were also relatively similar at 0.044ppm and 0.049ppm. The concentration of Fe in WC was lower in comparison, at 0.011ppm.

Table 5.1. Percentage composition of Aluminium, Manganese, Iron, Zinc and Lead as well as exchangeable acidity levels within the discard coal, weathered coal and soil utilised in studies.

Substrate	**Al (ppm)**	**Mn (ppm)**	**Fe (ppm)**	**Zn (ppm)**	**Pb (ppm)**	**Exchangable Acidity**
Hard Coal	0.061	<0.00005	0.044	<0.00005	<0.00005	-
Weathered Coal	0.079	<0.00005	0.011	<0.00005	<0.00005	12.8
Soil	0.087	<0.00005	0.049	<0.00005	<0.00005	1.3

Concentrations (ppm) of Arsenic (As) were highest in the HC (10.7ppm) followed by 4.75ppm in the soil. The As concentration in WC coal (0.38ppm) was substantially lower than the concentration in HC. Concentrations of Cd in HC, WC and soil were 0.14ppm, 1ppm and 0.23ppm and concentrations of Hg 2.2ppm, 2.4ppm and 0.33ppm respectively. The exchangeable acidity of the WC was notably higher than the exchangeable acidity of the soil used in trials. Exchangeable acidity analysis was not measured on the HC used in trials.

Table 5.2. Concentration of Arsenic, Cadmium, Mercury, Calcium, Potassium, Magnesium, Sodium and Phosphorus concentrations within the hard coal, weathered coal and soil utilised in studies.

Substrate	**As ppm**	**Cd ppm**	**Hg ppm**	**Ca ppm**	**K ppm**	**Mg ppm**	**Na ppm**	**P ppm**
Hard Coal	10.7	0.14	2.2	-	-	-	-	-
Weathered Coal	0.38	1	2.4	195	49.5	93.5	92	406.4
Soil	4.75	0.23	0.33	165	38.5	47.5	40.5	294.4

Percentage composition of sulphur (S) in substrates utilised were relatively low. The highest concentration of S measured, 1%, was in the HC. Percentage concentrations in WC and soil were both 0.1%. As may be expected, C concentrations increased within each substrate in comparison. A C concentration of 40.1% was measured in the HC and this reduced to 34.7% in the WC. It was observed though that the soil utilised in trials had quite a low C percentage of 2.7%.

Table 5.3. Percentage composition of Sulphur and Carbon within the discard coal, weathered coal and soil utilised in studies.

Substrate	**S %**	**C %**
Hard Coal	1	40.1
Weathered Coal	0.1	34.7
Soil	0.1	2.7

5.3.7 Elemental Analysis of Plant Material

Metal concentrations in plant material grown in HC amended with a WC supplement and subjected to various treatments are reported in Table 5.4, Table 5.5 and Table 5.6.

Concentrations of Cd, Zn and Hg in the roots and shoots of plants harvested from the treatment HC + WC + *C.dactylon* were found to be negligible (Table 5.4). The concentration of Mn within the shoots of these plants was also non-existent, and very low within the roots of these plants (0.005ppm). Pb concentrations in the shoots of these plants were also at non-detectable levels, but increased to 0.046ppm in the plant roots. The metal with the highest concentration within plants was Fe. In plant roots, a concentration of 6.268ppm was recorded. This was reduced to 4.169ppm in the plant shoots. The Al concentration within the roots and shoots of these plants was 2.213ppm and 1.782ppm respectively and concentrations of As 0.092ppm and 0.068ppm (Table 5.4).

Table 5.4. Selected metal concentrations within the roots and shoots of *Cynodon dactylon* plants grown on hard coal amended with a weathered coal supplement. Concentrations of metals within the soil utilised in trials, utilised in conventional dump cladding techniques, are also listed.

	Cd ppm	**Fe ppm**	**Pb ppm**	**Mn ppm**	**Zn ppm**	**As ppm**	**Al ppm**	**Hg ppm**
Shoot	0	4.169	0	0	0	0.068	1.782	0
Root	0	6.268	0.046	0.005	0	0.092	2.123	0
Soil	0.010	3.784	0.020	0	0	0.046	4.213	0.0052

Within the roots and shoots of plants harvested from the treatment HC + WC + *C.dactylon* + AMF, Mn and Zn concentrations were once again nil. Also non-detectable within the roots and shoots of plants were Cd and Pb (Table 5.5). The As concentration in the roots of these plants was 0.024ppm but dropped to 0 within the plant shoots. In comparison to *C.dactylon* plants grown on HC supplemented with WC and without mycorrhizal inoculation, AMF inoculated *C.dactylon* plants had lower Al and Fe concentrations. The Al concentration in the roots of these plants was 1.650ppm and dropped slightly to 1.332ppm in the plant shoots. Similarly, the 2.481ppm Fe concentration in the roots of these plants dropped to 1.467ppm in the shoots (Table 5.5). Reductions of around 25% for Al and approximately 65% for Fe were observed from non-mycorrhizal to mycorrhizal plants.

Table 5.5. Selected metal concentrations within the roots and shoots of *Cynodon dactylon* plants grown on hard coal amended with a WC supplement and inoculated with arbuscular mycorrhizal fungi. Concentrations of metals within the soil utilised in trials, utilised in conventional dump cladding techniques, are also listed.

	Cd ppm	**Fe ppm**	**Pb ppm**	**Mn ppm**	**Zn ppm**	**As ppm**	**Al ppm**	**Hg ppm**
Shoot	0	1.467	0	0	0	0	1.332	0
Root	0	2.481	0	0	0	0.024	1.650	0.000034
Soil	0.010	3.784	0.020	0	0	0.046	4.213	0.0052

Analysis of plants from the remaining treatment, HC + WC + *C.dactylon* + AMF + ECCN84, showed that Cd, Zn and Mn concentrations within the roots and shoots of plants were once again non-existent. Pb concentrations in plant roots were low at 0.014ppm and non-existent within plant shoots. In comparison to concentrations obtained in *C.dactylon* plants inoculated with AMF, As concentrations were higher in the roots and shoots of these plants at 0.032ppm and 0.008ppm respectively. Also higher in comparison were Fe and Al concentrations. The concentration of Al recorded in the roots of these plants was 1.712ppm and 1.590ppm in the plant shoots. The Fe concentration in the roots of these plants was 3.682ppm and in the shoots of these plants, 2.135ppm (Table 5.6).

Table 5.6. Selected metal concentrations within the roots and shoots of *Cynodon dactylon* plants grown on hard coal amended with a weathered coal supplement and inoculated with arbuscular mycorrhizal fungi and the EBRU Culture Collection Isolate Number 84. Concentrations of metals within the soil utilised in trials, utilised in conventional dump cladding techniques, are also listed.

	Cd ppm	**Fe Ppm**	**Pb ppm**	**Mn ppm**	**Zn ppm**	**As ppm**	**Al ppm**	**Hg ppm**
Shoot	0	2.135	0	0	0	0.008	1.590	0
Root	0	3.682	0.014	0	0	0.032	1.712	0
Soil	0.010	3.784	0.020	0	0	0.046	4.213	0.0052

5.4 Discussion

5.4.1 Humic Acid

Humic acid is a naturally occurring polyelectrolyte which occurs widely in soil, natural waters, lake and sea sediments as a result of the biological transformation of plant and animal residue in the environment (Lorenc-Grabowska and Gryglewicz, 2004). As humic substances are primarily the products of microbiological transformation of lignin and other plant debris, humic acid is also readily present within coal (Novák *et al.*, 2001). Coal humic acids can be described as dark coloured alkali soluble substances that occur naturally in some lignites and brown coals but are present in limited concentration in bituminous coals (Skhonde *et al.*, 2006). Humic acids present in coals differ from each other according to the grade (coalification) of the coal as well as the conditions under which the coal was formed (Kurková *et al.*, 2004). Humic acids form the major constituent of coal organic oxidation products and are formed on the surface of the coal substrate and are composed of various molecular weights (Skybová *et al.*, 2007).

Humic acid detection within columns was interpreted as a reflection of HC degradation. Alongside volatile fatty acids and methane, humic acid is one of the major products of coal breakdown with coal solubilisation by fungi yielding a variety of large molecules, principally humic acids (Senesi and Milano, 1994; Catcheside and Ralph, 1999; Machović, 2000). Humic acid analysis within columns (Figure 5.2) revealed that the treatment of HC + WC + *C.dactylon* + AMF + ECCN84 yielded the highest amounts of humic acid in comparison to the HC control. Via mechanical and chemical weathering, mainly through the exudation of compounds such as low molecular weight organic acids, mycorrhizal fungi have the ability to weather such material as rock to obtain primary minerals (van Breemen *et al.*, 2000a; Landerweert *et al.*, 2001). The addition of AMF to this treatment most likely enhanced coal oxidation within the column leading to higher levels of humic acid. Further coal oxidation could have occurred due to the action of ECCN84 which possesses the ability to oxidise and solubilise coal (Igbinigie *et al.*, 2007). The highest amount of humic acid production thus most likely resulted due to the combined effect of plant root, AMF and ECCN84 activity fungi within the coal substrate.

The relatively consistent level of humic acid down the length of control HC columns demonstrated that coal oxidation in the absence of *C.dactylon* did not take place. Column harvest also showed that the majority of plant roots formed in the upper sections of each treated column and gradually thinned down the length of the column indicating a correlation between root growth and coal breakdown. The weathering of rock by plant roots has been well documented and like mycorrhizal weathering, this phenomenon is linked to the need of plants to obtain minerals and nutrients (Berner and Cochran, 1998; Akter and Akagi, 2005).

5.4.2 Total Organic Carbon

The largest global pool of organic matter can be found in soils with the organic matter primarily comprising of a mixture of plant and animal remains in various decompositional stages. Also included in this pool are substances produced from the microbial or chemical synthesis of breakdown products, substances exuded from plant root systems as well as the remains and viable biomass of soil microorganisms (Schnitzer *et al.*, 1991; Jobbagy and Jackson, 2000). A great influence on the physical, chemical and biological properties of soils is exerted by soil organic C (Schlesinger, 1990).

TOC analysis down column lengths was undertaken to measure the amounts of C available within the rhizosphere as a result of each treatment that could in turn influence microbial growth. Litter inputs and organic substances released in to the rhizosphere by plants in the form of root exudates, lysates and sloughed-off cells support rhizospheric microbial communities typically resulting in increased population densities and increased activity (Curl and Truelove, 1986; Lynch and Whipps, 1990; Hodge *et al.*, 1998).

Clear increases in organic C were observed in treated columns in comparison to the HC control (Figure 5.3). Treated columns would have had a higher input of rhizospheric C, supplied by *C.dactylon*, which not have occurred within the HC control columns. Simultaneously, the biodegradation of the coal substrate occurring in treated columns would have increased C levels within the rhizosphere. For this reason, it may be expected that differences in organic C concentrations between treated columns and the HC control should have reflected coal breakdown, and this correlate with humic acid formation. This however was not observed. Though TOC levels within the treatment HC + WC + *C.dactylon* were comparable, substantial reductions in TOC levels were observed in the presence of AMF and ECCN84. The hyphal binding, and possible utilisation, of humic acids by fungal hyphae has been observed (Igbinigie, 2007). TOC concentrations were measured using samples containing water soluble organic C whereas humic acid concentrations were determined by extraction. The method of extraction most likely increased humic acid concentrations such that substantially higher levels were recorded in comparison to soluble organic C levels obtained. These levels would have been reduced by the presence of AMF and ECCN84 in comparison to when they were absent, as was observed. Soluble organic C in the form of root exudates would have also been reduced through utilisation by both AMF and especially ECCN84. The slightly higher levels of TOC observed in the treatment of HC + WC + *C.dactylon* + AMF in comparison to the treatment HC + WC + *C.dactylon* + AMF + ECCN84 may have been influenced by organic acid exudation by the mycorrhizal plants, with such excess C being utilised in the presence of ECCN84.

5.4.3 Moisture

Acid mine drainage forms by the oxidation of sulphide minerals in coal deposits worldwide and is a serious environmental problem faced by the mining industry (Zhao *et al.*, 2006). Pyrite and maracasite are the minerals primarily responsible for acid mine drainage in coal due to their high acid-forming metal sulphide component (Pinetown *et al.*, 2007). The utilisation of water during the process of coal mining and rehabilitation combined with pyrite oxidation and acid formation (H_2SO_4) often results in the build up of aquifers containing significant amounts of highly acidic water (Tabaksblat, 2002; Pinetown *et al.*, 2007). The ingress of water as well as atmospheric oxygen into coal discard dumps combine in the formation of acid mine drainage. Cladding of coal discard dumps is designed to reduce the ingress of water and oxygen.

Moisture content was measured to provide some indication of the extent of water ingress into the coal substrate, with treated columns compared to HC control columns. At the 20cm depth, variation in moisture content across treatments was observed (Figure 5.4) in comparison to the HC control. Moisture content percentages in the treatments HC + WC + *C.dactylon* and HC + WC + *C.dactylon* + ECCN84 here were significantly higher than in the HC control indicating water retention in this zone. Visual analysis of harvested columns did show that the 0-20cm section of treated columns did retain a substantial amount of moisture in comparison to the sections below.

Below the top 20cm sections of each column however, moisture content percentages in all treated columns were lower than that of the untreated HC control indicating reduced ingress most likely due to *C.dactylon* utilisation and evapotranspiration, primarily. For plants inoculated with AMF, water uptake would have been enhanced due to the increased absorbance area of roots provided by the outgrowing hyphae in the rhizosphere (Ahmad Khan *et al.*, 2003; Bolandnazar *et al.*, 2007).

5.4.4 Oxygen

The self-heating and spontaneous combustion of coal have also posed serious issues for the coal mining industry (Sujanti and Zhang, 1999). Not only does spontaneous combustion lead to the loss of product as well as the desired properties of coal, it also raises serious safety and environmental concerns (Denis *et al*, 2007). The exposure of coal to air and water that results in the ignition of coal through processes of chemisorption, oxidation and spontaneous combustion is a global concern with burning coal having the potential to cause a range of environmental problems (Bell *et al.*, 2001; Nolter and Vice, 2004; Stracher and Taylor, 2004; Whitehouse and Mulyana, 2004). It has been estimated that 1t worth of typical coal mine waste has the potential to produce 0.84kg sulphur dioxide (SO_2), 0.61kg hydrogen sulphide (H_2S), 0.03kg nitrogen oxide (NOx), 99.7kg carbon monoxide (CO) and 0.45 kg smoke (Liu *et al.*, 1997). This is especially an issue with coal discard dumps which still posses 5-15% residual coal (Carpentier *et al.*, 2005). The porous nature of such dumps enables air and water to circulate within the dumps increasing the likelihood of spontaneous combustion. Spontaneous combustion of coal occurs when the quantity of heat generated by the oxidation of organic matter is greater than the quantity of heat dissipated (Misra and Singh, 1994). Even though auto-oxidation of the organic element of coal leading to self-ignition is the prime cause of spontaneous combustion (Banerjee, 1981), the storage of coal can provide a new set of factors that exert an influence such as heat capacity, thermal conductivity, bulk density, configuration of the stockpile and climatic conditions (Misra and Singh, 1994).

The measurement of oxygen in the column study showed some reduction in oxygen levels within the coal substrate across all treatments in comparison to the HC control. Although not significantly, it was apparent that *C.dactylon* growth reduced oxygen levels within the discard HC substrate due to either the reduction of oxygen ingress or through oxygen utilisation within the coal substrate, or possibly both.

The majority of studies conducted on soil respiration, an indicator of microbial activity, have mainly focussed on soil C pools (Raich and Schlesinger, 1992; Keith *et al.*, 1997). What is clear from these studies is that alongside significant levels of CO_2 efflux within soils, due to plant and microbial respiration, significant levels of oxygen uptake takes place as well. A large majority of soil dwelling microorganisms, both beneficial and pathogenic, are aerobic and thus alongside essential elements such as nitrogen, require oxygen for growth and activity (Myrold and Posavatz, 2007). Similarly, in plants, respiration is essential for the growth and activity of plant tissues with solar energy conserved during the process of photosynthesis released in a regulative manner for the production of Adenosine triphosphate (ATP) (Gonzales-Meler *et al.*, 2004). Plants may translocate an estimated 35-80% of photosynthetic C below ground for root production and respiration, mycorrhizas and root exudates (Davidson *et al.*, 2002; Giardina *et al.*, 2003).

Though results obtained from column experiments were indicative of oxygen levels within columns that underwent different treatments, the method by which oxygen readings were recorded could have been more accurate and less invasive. Techniques utilised here were restricted by the equipment used as well as modes of sampling the columns. The thrusting of the oxygen probe into the coal substrate from the top of each column could have introduced atmospheric oxygen into the coal and influenced results. More accurate results may have been obtained had dedicated ports been used in columns, established at trial initiation. However, the trend of results indicates that the degraded coal layer generated by *C.dactylon*/fungal activity does act to reduce oxygen ingress to some measure.

5.4.5 pH

The effective treatment of overburden material from open-cast coal mining is important in minimising the potential for acid-mine drainage formation. As previously described, processes involved in coal mining expose large amounts of iron-sulphide containing minerals such as pyrite to oxygen and water with exposure often resulting in oxidation and hydrolysis reactions that produce sulphuric acid (Gerke *et al*., 1998). Sulphuric acid produced from acid mine drainage from waste coal material can have pH values below 1, and in conjunction with high levels of sulphate and soluble metals also derived from the coal, acid mine drainage can be a serious environmental risk to water quality, natural biota as well as human health (Herlihy *et al*., 1990; Short *et al*., 1990; Elders, 2001). pH is thus a good indicator of both moisture and oxygen into the dump system. Currently, the majority of mining operations in South Africa utilise neutralisation to counteract the issue of acid mine water formation with lime often utilised as a neutralising agent (Maree *et al*., 2004a).

pH was measured down the length of each column and significantly higher pH measurements were observed in treated columns in comparison to the untreated HC control column at the top of the column. pH levels gradually became similar across all columns in the middle and bottom sections of each column (Figure 5.6). In the top 20cm of each column, the treatment of HC + WC + *C.dactylon* + AMF + ECCN84 yielded the most alkaline pH levels in comparison to the HC control suggesting that this treatment best reduced water and oxygen ingress into the column, thus reducing sulphuric acid formation. This was observed to be the case for water ingress in across treated columns. AMF or ECCN84 may also release basic compounds that could have affected pH levels, compounds such as glomalin in the case of AMF.

5.4.6 Elemental Analysis of Coal and Soil

Elemental analysis of the substrate utilised in studies indicated further conditions that have limited plant growth in WC supplement in comparison to soil, this demonstrated in pot studies (Section 2.3.1). Alongside growth limiting factors such P, Ca and Mg deficiencies and Al and Mn toxicity, soil acidity is one of the most important environmental factors that can affect plant growth and can acutely limit crop production (Beegle and Lingenfelter, 1995) Soil acidity is composed of two components, active acidity and exchangeable acidity (Beegle and Lingenfelter, 1995). The concentration of H^+ ions in the solution phase of the soil, measured in pH, is referred to as the active acidity of soil and is not the measure of total soil activity. The exchangeable (reserve) acidity of soils refers to the amount of H^+ ions on cation exchange sites of negatively charged clay and organic matter fractions of soil (Beegle and Lingenfelter, 1995). The exchangeable acidity of WC, a fraction of the WC supplement, was observed to be significantly higher than in soil and this may have limited plant growth. Levels of essential elements were comparable with and often higher than levels within the soil substrate utilised in trials, and this would not have been limiting. Analysis also showed that C concentrations in the WC were also substantially higher than in the soil. Glomalin production may have also been further reduced in the presence of the WC supplement, an occurrence often associated with high C soils (Comis, 2002).

5.4.7 Elemental Analysis of Plant Material

The ability of plants to tolerate acidic and toxic conditions varies widely with species (Strock, 1998). It has been established that at pH values below pH 3, plants are negatively affected by ph directly (Arnon and Johnson, 1942). However, indirect effects of low pH levels can also greatly limit plant growth. Within discard dumps, the oxidation of pyritic material and the formation of acids results in the increase of solubility and availability of a variety of metals, primarily Al and Mn (Strock, 1998). These two metals are considered to be elements that most often limit plant growth on strip mines when present at relatively high concentrations (Fail and Wochok, 1977).

Toxic concentrations of Al inhibit root growth and interfere with the uptake of P, an essential plant nutrient (Foy *et al.*, 1978). The majority of plants require an Al concentration of no more than 200ppm for adequate performance (Mosser-Pietraszewska, 2001). Root and shoot concentrations of Al within *C.dactylon* plants sampled from across the series of treatments were substantially lower than levels that would cause toxicity (Tables 5.4 to 5.6). Al concentrations were further reduced in plants inoculated with AMF (Tables 5.5 and 5.6).Mycorrhizal fungi have been shown to enhance plant resistance to Al (Cumming and Ning, 2003; Kelly *et al.*, 2005; Rufykiri *et al.*, 2000). It is likely that this was the case in the treatments involving column inoculation with AMF. Simultaneously, the higher levels of this metal within plant roots would suggest a higher accumulation in this section of plants, with accumulation of metals by AMF having also shown to be a method by which AMF enhance Al resistance (Cumming and Ning, 2003).

Concentrations of alternate non-essential heavy metals such as Pb, Cd and Hg were negligible within plant roots and shoots and thus much lower than levels plants are able to tolerate as well as levels that would result in toxicity (Beauford *et al.*, 1977; Kabata-Pendias and Dudka, 1991). Hg, normally found between 0.001 and 001ppm in plants (Gracey and Stuart, 1974), at high concentrations has been shown to interfere with photosynthesis and transpiration (Schlegel *et al.*, 1987), water uptake (Beauford *et al.*, 1977) and chlorophyll formation (Prasad and Prasad, 1987).

Concentrations of essential mineral nutrients required for plant growth were observed to be relatively low for concentrations considered adequate in higher plants. Fe, an essential element in chlorophyll synthesis is usually found at 100ppm concentration, Mn, required for chloroplast membrane integrity and for oxygen release in photosynthesis is usually found at a concentration of 50ppm and Zn, a component of many plant enzymes, is usually found at 20ppm (Salisbury and Ross, 1978; Raven *et al.*, 1992). Concentrations of these elements in plant roots and shoots were substantially lower, most likely indicative of limited nutrient availability in discard HC dumps and the importance of AMF associations.

5.5 Conclusions

- The simulation of the coal dump environment in the column study confirmed previous field observations and showed that HC is degraded to a comparable humic soil-like substrate in the presence of *C.dactylon* growth. The oxidation of coal to humic acids within the *C.dactylon* rhizosphere was further enhanced by the presence of AMF and ECCN84.

- Reductions of organic C in the coal substrate containing *C.dactylon*/AMF/ECCN84 growth coinciding with increases in humic acid concentrations suggests a binding, and possible utilisation, of this available C by the fungal populations.

- Moisture and oxygen ingress reductions coupled with increases in pH levels within columns treated with a WC supplement and subjected to *C.dactylon* growth suggest that the *in situ* generation of a degraded coal soil-like layer offers a possibility for dump rehabilitation.

- If this method of dump rehabilitation was to be further developed, the accumulation of heavy metals at toxic concentrations within plants does not appear to present a serious problem.

CHAPTER 6:

CONCLUSIONS

6.1 The Role of Arbuscular Mycorrhizal Fungi in the Biodegradation of Coal

Although the fundamental processes involved in the biotransformation of coal have been thoroughly examined, there have been limited large-scale industrial applications utilising such systems (Klein *et al*., 1999). During an investigation into the processes of biotransformation occurring in South African mine dumps, Rose (pers. comm. 2005) reported the un-anticipated occurrence of sporadic *C.dactylon* growth directly on the surface of un-rehabilitated HC discard dumps including dumps at the Anglo Coal Navigation Mine (Witbank, Mpumalanga). An extensive growth of plant roots below the HC surface was observed in areas of *C.dactylon* growth and the coal was found to have been broken down into a humic soil-like material. Trenches dug in the vicinity of the *C.dactylon* growth revealed extensive root establishment and it was primarily the top 1.5m of the dump in which the soil-like substrate formation had occurred.

Extensive fungal growth was observed to be associated with the *C.dactylon* root system with fungal hyphae extending deep into the surrounding coal substrate. Preliminary investigations, including the staining of *C.dactylon* root sections, further revealed the extensive colonisation of these plants by mycorrhizal fungi. From these observations, it was evident that a complex microbial environment had established in the rhizosphere of the *C.dactylon* plants. From initial results obtained, it was proposed that the breakdown of coal observed here could involve a two-part biological system including the plant/mycorrhizal association on the one hand as well as populations of non-mycorrhizal fungi operating in the rhizosphere on the other. The objective of the study reported here was to test this hypothesis and to focus specifically on the plant/mycorrhizal system.

Simultaneously with this study, Igbinigie (2007) had investigated the rhizosphere of the *C.dactylon*/coal system and the studies were collectively known as the Fungcoal Project, a study sponsored by the Anglo Coal Corporation. Extensive rhizosphere sampling and screening studies of the coal dump environment undertaken by Igbinigie *et al.* (2007) reported the isolation of over 120 fungal isolates with coal biodegrading properties. Total genomic analysis revealed the presence of species from the genera *Trichoderma*, *Penicillium* and *Aspergillus* genera which had been previously reported to demonstrate coal biodegradation capabilities (Hölker *et al.,* 2002; Achi, 2004; Pokorný *et al*., 2005). A newly reported isolate in the coal environment, *N.fischeri* (ECCN84)**,** was identified which could efficiently degrade HC resulting in the formation of humic acids and a range of aromatic compounds.

The study of the plant/mycorrhizal component of the *C.dactylon*/coal system, reported here, was undertaken at the same time as the study reported by Igbinigie *et al.* (2007) in order to investigate the role played by mycorrhizal fungi in the coal biodegradation process. The objective was to interrogate the hypothesis that interactions between *C.dactylon*, associated mycorrhizal fungi and non-mycorrhizal fungi growing in the rhizosphere of the *C.dactylon* root system acted synergistically to enhance the breakdown of coal into a humic soil-like material. The study undertook the identification of species of mycorrhizal fungi present in the plant/fungal system from which a mycorrhizal inoculum could be prepared. The coal dump environment was then replicated under controlled laboratory conditions and the effects on coal breakdown of the associated mycorrhizal and non-mycorrhizal rhizospheric fungi present associated with *C.dactylon* growth was measured.

Initial pot trial studies confirmed that the process of *C.dactylon* establishment on HC was probably driven by a dual fungal system involving mycorrhizal fungi and associated non-mycorrhizal rhizospheric fungi. A key finding from this experiment suggested that in the absence of soil, the extent of extraradicular mycorrhizal establishment was reduced and internal plant root colonisation enhanced. The results suggested that the coal biosolubilising ability of the ECCN84 isolate used in this study may provide increased amounts of nutrients to both plant and mycorrhizal fungi which may have resulted in the reduction of extraradicular mycorrhizal establishment observed. In return, the increased mycorrhizal fungal colonisation of the *C.dactylon* root system may indicate an increased requirement to provide organic C in the form of exudates to enable the rhizospheric fungi to function in such a harsh environment and provide the necessary nutrients. Previous studies have shown the dependence on organic supplementation of fungi involved in HC biotransformation (Klein *et al*., 1999; Igbinigie *et al*., 2007).

Characterisation studies of the mycorrhizal inoculum developed for this study showed the presence of the species *Gi.gigantea*, *G.clarum*, *P.occultum* and *G.mosseae*. On completion of pot trials, *P.occultum* was re-isolated from the degraded coal substrate thus satisfying at least 3 of Koch's postulates and indicating a causal role for AMF in the system. Also re-isolated was ECCN84.

The discard coal dump environment was simulated in column experiments and results indicated clear structural changes of the coal substrate occurred where it was exposed to *C.dactylon* growth in association with AMF and ECCN84, compared to controls. The HC was converted to a humic soil-like substrate comparable to that observed in the coal dumps colonised by *C.dactylon* over the period of 44 weeks that the study ran. SEM studies of the substrate showed a possible role for fungal hyphal weathering of coal with hyphal penetration of coal particles and fracturing along the penetration zones. This was shown to be linked with the formation of low molecular weight organic acids. These results were compared to observations made by Jongmans *et al* (1997) who observed similar weathering of minerals by ectomycorrhizal fungal hyphae which was popularised as the concept of "rock-eating fungi". The account of "coal-eating fungi" operating in this system may be a new description of the scope of the fungal weathering process. However, if the observations are correct that extraradicular mycorrhizal establishment is limited and that the task of weathering and the associated recovery of nutrients is delegated to non-mycorrhizal coal-degrading fungi influenced by the plant/mycorrhizal system, this may add to insight on how the process operates. Confirmation of this proposal will require further follow-up work.

Chemical analysis of the degraded coal substrate in the simulation studies further supported the idea that the process of *C.dactylon* establishment on HC and the soil-like formation of the substrate was enhanced by the dual-fungal system of mycorrhizal and non-mycorrhizal rhizospheric fungi. Increases in extractable humic acid were observed in the presence of *C.dactylon* in association with populations of the two fungi, however, interestingly, this was coupled with reductions in free soluble organic C. Results mirrored observations by Igbinigie (2007) who observed binding of free humic acid by fungal hyphae in such systems.

6.2 Descriptive Model

Based on the results reported for both the mycorrhizal and non-mycorrhizal components of the *C.dactylon*/coal system, a putative descriptive model was developed to explain the mechanisms by which the coal weathering in the system could occur (Figure 6.1). Results suggest that the processes observed are primarily driven by the translocation of photosynthetic organic C into the rhizosphere with the key component of the system being the *C.dactylon* plant. This is mediated, and possibly controlled, by the associated AMF. The exudate in the rhizosphere could then play either a direct or an indirect role in the oxidative weathering of coal. Organic C utilised by AMF could be directly converted to organic acids, amongst other compounds, and exuded by these fungi. Indirectly, such exudates could be utilised by coal solubilising fungi such as ECCN84 as an organic C source which are then able to utilise a number of reported strategies to degrade the coal substrate. In return, nutrients released from the coal substrate are returned to the plant. The ultimate result of this interactive process would be the generation of the humic soil-like material derived from HC substrate. A schematic diagram of the proposed model is shown in Figure 6.1. Though the majority of previous studies investigating the extent of the mycorrhizosphere have indicated that it exceeds the associated plant's own rhizosphere, this study suggests that in this case the area of mycorrhizal influence is reduced in the coal environment in the presence of coal-degrading rhizospheric fungi, as indicated in the diagram. It seems probable that here the total zone of rhizospheric influence exceeds the zone of mycorrhizal influence.

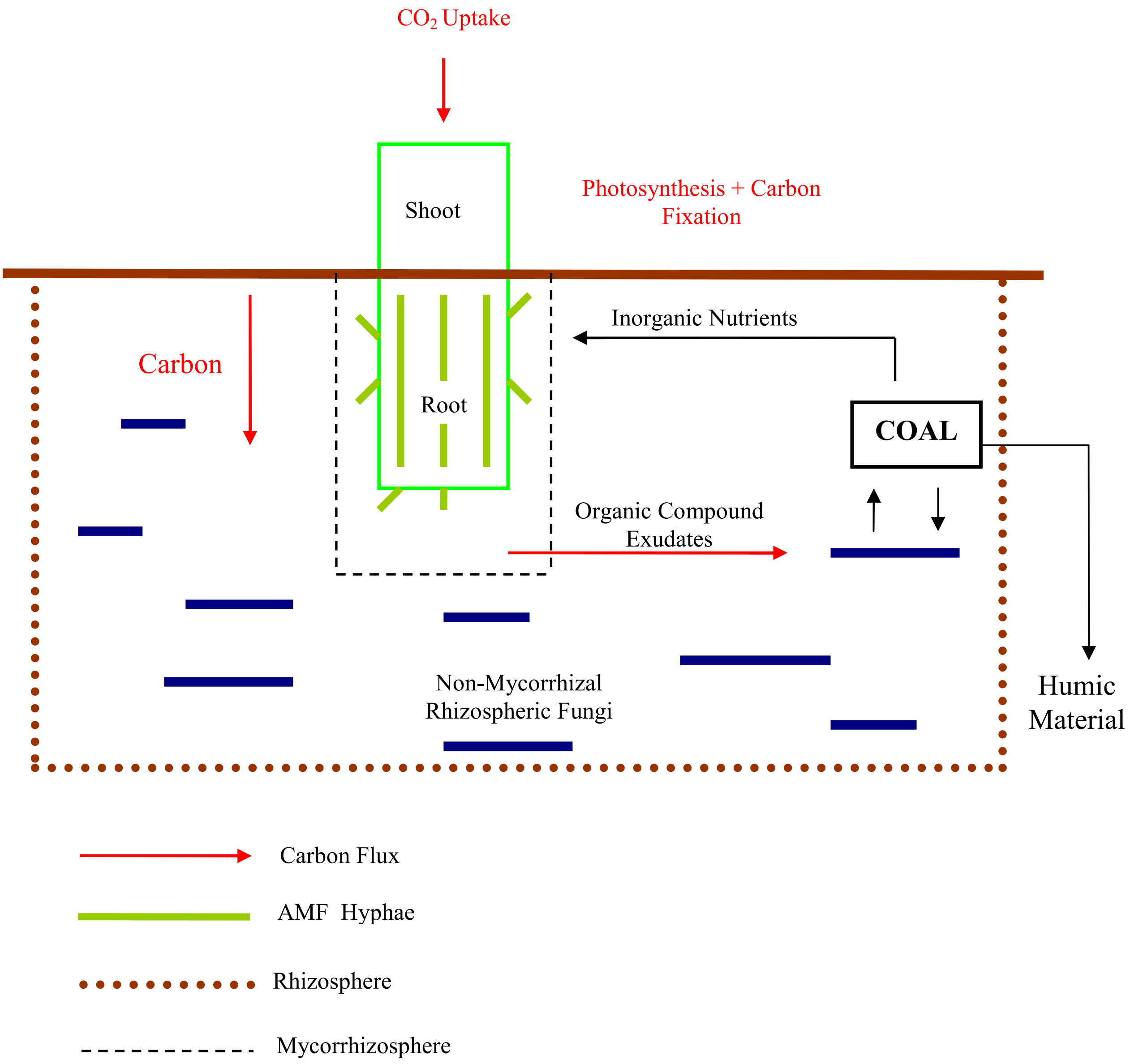

Figure 6.1. Schematic diagram illustrating the possible association between *Cynodon dactylon,* arbuscular mycorrhizal fungi and rhizospheric organisms that leads to the degradation of coal *in situ*.

6.3 Future Work

The results reported here are first descriptions of the system and substantial further work will be required to confirm the observations made and should include the following investigations:

- This study focussed primarily on the role of fungal species in the biodegradation of coal, however bacteria, archaea and protozoa are present in the rhizosphere and their role may possibly influence this system and should be investigated.

- Although glomalin analysis utilising a protein assay did provide an indication of extraradical hyphal establishment, the utilisation of the monoclonal glomalin antibody Mab32B11 in an ELISA would have enhanced the accuracy of detection.

- The utilisation of dual-chamber systems would enable the separation of hyphal and root growth which would further establish the extent of extraradicular hyphal establishment and confirm if this is indeed reduced in the presence of rhizospheric fungi such as ECCN84.

- The species of AMF colonising *C.dactylon* roots (intraradicular colonisation) needs to be investigated in greater detail.

- Koch's postulates need to be rigorously demonstrated with the recovery of inoculated AMF species from treated plants and the re-introduction of inocula back into the system producing the same effects.

- The utilisation of radiolabeled studies to describe C-flux through the system.

6.4 Industrial Applications

Concurrent with these investigations, field trails were established at the Kleinkopje Colliery discard coal dump. The biodegradation of HC was replicated in the presence of AMF and ECCN84 inocula (Horan, 2007). Experimental results contributed to the development of the "Stacked-Heap Coal Bioreactor" concept (Patent ZA 2007/07607) in which waste HC can be converted to 30-40% humic acids which may be recovered and used in a variety of ways including:

- Methane Production. Mutambanengwe (2007) has shown that the humic product can be converted to methane in the presence of an adapted anaerobic bacterial consortium.

- Fertiliser. The possibility of using the humic product as a fertiliser in the agricultural sector has also been investigated and enhanced growth of plants using the biodegradation product was demonstrated (Horan, 2007).

- Soil Rehabilitation. Horan also demonstrated that the humic product could be used to effect the decompaction of soils used in the rehabilitation of open-cast mining areas.

- Dump Cladding and Rehabilitation. The field studies noted above demonstrated that the system could be used to establish a self-cladding function on discard coal dumps. Currently, large volumes of topsoil are used to rehabilitate waste coal discard dumps and apart from dumps having a limited life-span due to erosion, substantial damage to the surrounding environment results. In the self-cladding operation, the coal surface would be converted to a humic soil-like substrate and in addition to stabilizing the dump surface could also control water and oxygen ingress into the system. Preliminary studies indicated that the successful application of the process and cost saving over topsoil translocation would reduce rehabilitation costs ~ 8 fold (Horan, 2007).

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APPENDIX 1:

CHAPTER 2

Alkaline Hydrogen Peroxide (Smith and Dickson, 1997)

3ml NH_4OH (Ammonia)
30ml 10% H_2O_2 (Keep in fridge)
567ml Distilled Water

Prepare in a fume cupboard. Prepare fresh solutions prior to use.

Lactoglycerol Trypan Blue Stain (Smith and Dickson, 1997)

Lactic Acid:Glycerol:Water (13:12:16)
520ml Lactic Acid
480ml Glycerol
640ml Distilled Water
0.82g Trypan Blue

Store in a dark bottle.

Lactoglycerol Trypan Blue Destain

As above without Trypan Blue stain.

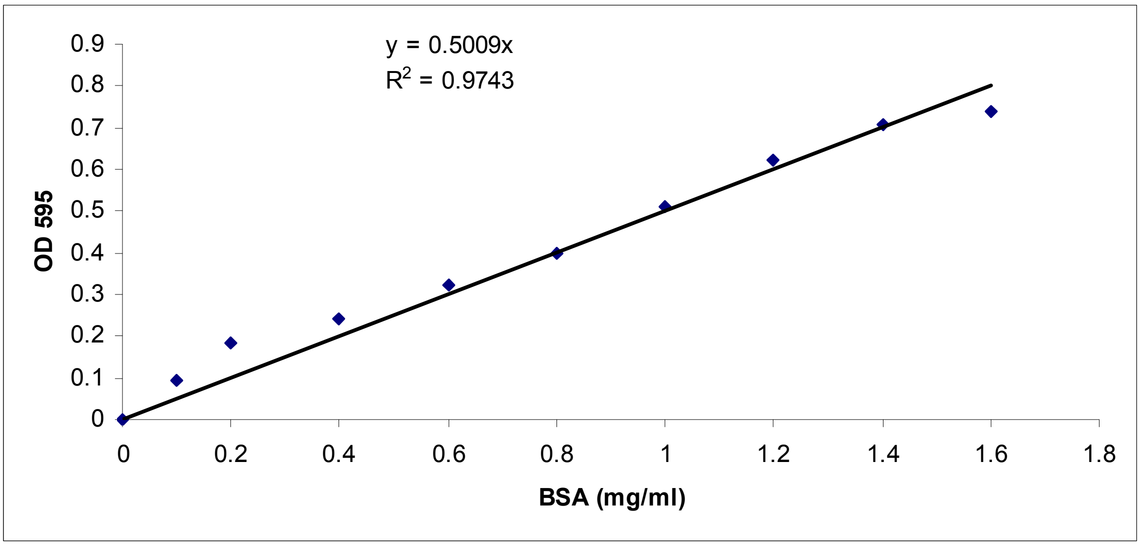
Store in a dark bottle.

Sodium Citrate (50mM) (Wright *et al*., 1996)

6.45g Sodium Citrate
500ml Distilled Water

Adjust pH with HCl.

Glomalin Standard Curve



APPENDIX 2:

CHAPTER 3

Melzer's Reagent

100g Chloral Hydrate
100ml Distilled Water
1.5 Iodine
5g Potassium Iodide

PVLG

100ml Distilled Water
100ml Lactic Acid
10ml Glycerol
16.6g Polyvinyl Alcohol

Phosphate Buffered Saline

8g Sodium Chloride
0.2g Potassium Chloride
1.44g Disodium Phosphate
0.24g Potassium Dihydrogen Phosphate
800ml Distilled Water

pH to 1.2 with Hydrochloric Acid and make volume up to 1000ml. Autoclave for 30 minutes.

Buffer A

2.42g Tris
1.86g EDTA
1.17g Sodium Chloride
0.058g Sodium Citrate
0.147 Calcium Chloride
100ml Distilled Water

Autoclave for 30 minutes.

Lysozyme (50mg/ml)

50mg Lysozyme Stock
1ml Autoclaved Distilled Water

Proteinase (50µg/ml)

50µg Proteinase K
1ml Distilled Water

Sodium Dodecyl Sulphate (10%)

10g Sodum Dodecyl Sulphate
100ml Distilled Water.

Prepare in fume hood Autoclave for 30 minutes.

Chloroform :Isoamyl alcohol (24:1)

96ml Chloroform
4ml Isoamyl Alcohol

Sodium Acetate (3M)

123.45g Sodium Acetate
500ml Distilled Water

Autoclave for 30 minutes.

TAE Buffer (0.5X)

50ml 50X TAE Buffer
100ml Distilled Water

Ethidium Bromide

0.5g Ethidium Bromide Powder
1ml Distilled Water

Dissolve and store safely in a dark bottle at room temperature.

DNA Loading Buffer (10X)

250mg Bromophenol Blue
33ml 150mM Tris (pH 7.6)
60ml Glycerol
7ml Water

λ*Pst1* Molecular Weight Marker

Digest 200µl λDNA (0.25µg/µl) with 24µl 10X buffer H and 10µl of *Pst1* enzyme for 3 hours at 37°C. Add 550µl 10mM TE buffer (pH 8) and 150µl 6X loading buffer. Aliquot 100µl into 1ml Eppendorf tubes and store at -20°C.

Band Sizes- λ*Pst1* Molecular Weight Marker (bp)

- 14057
- 5077
- 4749
- 4507
- 2838
- 2459
- 2443
- 2149
- 1986
- 1700
- 1159
- 1093
- 805
- 514
- 468
- 448
- 339
- 264
- 247
- 216
- 211
- 200
- 164
- 150

AM1 Primer (WhiteSci)

5’-GTTTCCCGTAAGGCGCCGAA-3’

NS31 Primer (WhiteSci)

5’-TTGGAGGGCAAGTCTGGTGCC-3’

Bovine Serum Albumin (50mg/ml)

50mg Bovine Serum Albumin
1ml Autoclaved Distilled Water

Aliquot into 1ml Eppendorf tube, filter sterilise and store at -20°C.

Dimethyl Sulphoxide (25%)

250µl Dimethyl Sulphoxide
750µl Autoclaved Distilled Water

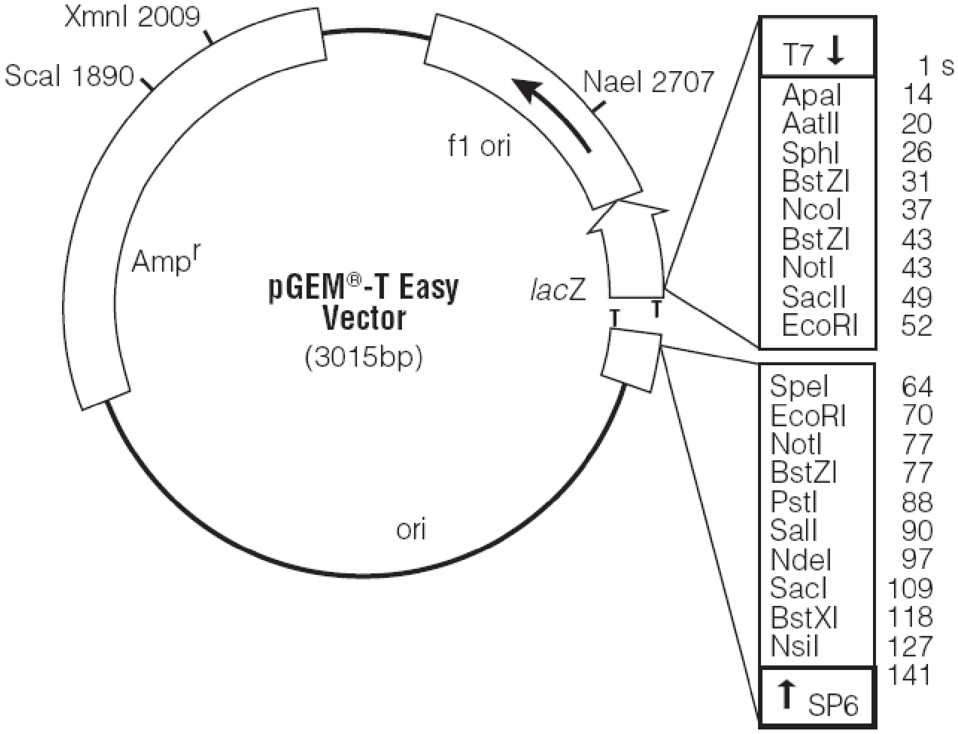
Aliquot into 1ml Eppendorf tube, filter sterilise and store at -20°C.

10mm DNTPs (Promega)

30µl dATP (U120D)
30µl dTGP (U123D)
30µl dCTP (U122A)
30µl dGTP (U121D)
180µl Distilled Water

Aliquot 60µl of mixtures into1ml Eppendorf tubes. Store at -20°C.

pGEM-T Easy Plasmid Vector Map



(http://www.promega.com/tbs/tm042/tm042.pdf)

RF1 Solution

7.4g Potassium Chloride
9.89g Manganese Chloride
7.94 Acetic Acid Potassium Salt
1.1g Calcium Chloride
850ml Distilled Water

150ml Glycerol

Autoclave glycerol and water separately before mixing on cooling. pH to 5.8 with Acetic Acid.

RF2 Solution

0.4g 4-Morpholine-propanesulfonic acid (MOPS)
0.15g Potassium Chloride
1.66g Calcium Chloride
170ml Distilled Water

30ml Glycerol

Autoclave glycerol and water separately before mixing on cooling. pH to 6.8 with Acetic Acid/Potassium Hydroxide.

Luria Bertani/Ampicillin Agar Plates

100mg Ampicillin
1ml Distilled Water

Add to 1L of Luria Bertani Agar.

IPTG (20mg/ml)

0.02g IPTG
1ml Distilled Water

Store at -20°C. Add 40µl per plate.

X-gal (100mg/ml)

0.1g X-Gal
1ml Dimethylformamide

Store at -20°C. Add 40µl per plate.

SP6 Primer (WhiteSci)

5'-ATTTAGGTGACACTATAGAA-3'

EDTA (125mM)

18.26g
500ml Distilled Water

Autoclave for 30 minutes.

ITS1-F Primer (WhiteSci)

5’- CTTGGTCATTTAGAGGAAGTAA-3’

ITS4 Primer (WhiteSci)

5’- TCCTCCGCTTATTGATATGC-3’

APPENDIX 3:

CHAPTER 4

Phosphate Buffer

35.8g/L Disodium Phosphate Dihydrate, Solution A
13.6g/L Potassium Dihydrogen Phosphate, Solution B

Mix solutions in ratio of 4 parts Solution A: 1 part Solution B.

Buffered Gluteraldehyde

10ml 25% ultrastructure grade gluteraldehyde
90ml Phosphate buffer

Store in a dark bottle at 4°C.

Sodium Hydroxide (1%)

1g Sodium Hydroxide
100ml Methanol

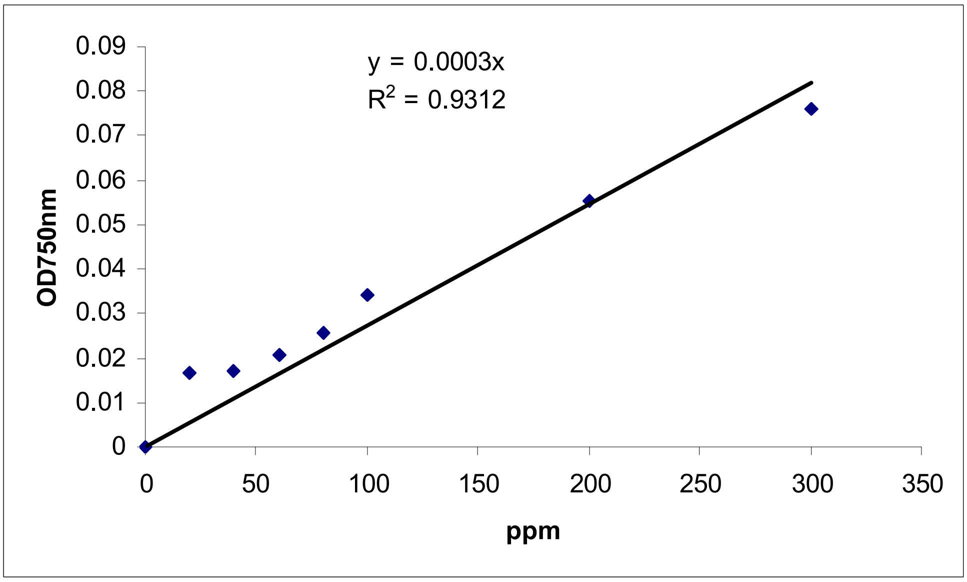
Sodium Bicarbonate (50mM)

2.1g Sodium Bicarbonate
500ml Distilled Water

APPENDIX 4:

CHAPTER 5

Humic Acid Standard Curve (Folin-ciocalteus)



Potassium Hydrogen Phthalate (2000ppm)

0.2g Potassium Hydrogen Phthalate
100ml Distilled Water

ICP-Elemental Standard Curve r^2 Values

Element	**Standard Curve r^2 Value**
Cd	0.987
Fe	0.991
Pb	0.995
Mn	0.988
Zn	0.995
As	0.992
Al	0.989
Hg	0.993

Table A4.1. Humic acid value standard errors at each point of analysis within treated and control columns. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control. Values in treated columns significantly different to values in the hard coal control column at the corresponding point are indicated in red.

Treatment	**Column Depth (cm)**	**Mean Value**	**Standard Error**
HC+ WC + *C.dactylon*	20	56.67	2.16
HC+ WC + *C.dactylon*	40	46.67	1.21
HC+ WC + *C.dactylon*	60	29.17	0.73
HC+ WC + *C.dactylon*	80	19.17	0.55
HC+ WC + *C.dactylon*	100	19.17	0.55
HC+ WC + *C.dactylon*	120	19.17	0.55
HC+ WC + *C.dactylon*	140	18.33	0.50
HC+ WC + *C.dactylon* + AMF	20	97.50	2.27
HC+ WC + *C.dactylon* + AMF	40	80.83	1.57
HC+ WC + *C.dactylon* + AMF	60	46.67	1.11
HC+ WC + *C.dactylon* + AMF	80	22.50	0.20
HC+ WC + *C.dactylon* + AMF	100	22.50	0.20
HC+ WC + *C.dactylon* + AMF	120	20.83	0.10
HC+ WC + *C.dactylon* + AMF	140	20.0	0.29
HC+ WC + *C.dactylon* + AMF + ECCN84	20	116.67	3.74
HC+ WC + *C.dactylon* + AMF + ECCN84	40	90.83	2.59
HC+ WC + *C.dactylon* + AMF + ECCN84	60	50.83	0.86
HC+ WC + *C.dactylon* + AMF + ECCN84	80	34.17	1.40
HC+ WC + *C.dactylon* + AMF + ECCN84	100	34.17	1.04
HC+ WC + *C.dactylon* + AMF + ECCN84	120	30.83	0.95
HC+ WC + *C.dactylon* + AMF + ECCN84	140	31.67	0.94
HC Control	20	18.17	0.13
HC Control	40	22.50	0.10
HC Control	60	20.0	0.24
HC Control	80	21.83	0.13
HC Control	100	18.33	0.12
HC Control	120	21.67	0.12

Table A4.2. Total organic carbon value standard errors at each point of analysis within treated and control columns. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control. Values in treated columns significantly different to values in the hard coal control column at the corresponding point are indicated in red.

Treatment	Column Depth (cm)	Mean Value	Standard Error
HC+ WC + *C.dactylon*	20	39.35	0.17
HC+ WC + *C.dactylon*	40	38.55	0.13
HC+ WC + *C.dactylon*	60	36.31	0.16
HC+ WC + *C.dactylon*	80	33.70	0.27
HC+ WC + *C.dactylon*	100	28.52	0.12
HC+ WC + *C.dactylon*	120	23.51	0.10
HC+ WC + *C.dactylon*	140	19.55	0.08
HC+ WC + *C.dactylon* + AMF	20	17.44	0.25
HC+ WC + *C.dactylon* + AMF	40	17.69	0.21
HC+ WC + *C.dactylon* + AMF	60	18.40	0.24
HC+ WC + *C.dactylon* + AMF	80	20.01	0.11
HC+ WC + *C.dactylon* + AMF	100	18.29	0.11
HC+ WC + *C.dactylon* + AMF	120	16.99	0.04
HC+ WC + *C.dactylon* + AMF	140	15.87	0.03
HC+ WC + *C.dactylon* + AMF + ECCN84	20	20.89	0.60
HC+ WC + *C.dactylon* + AMF + ECCN84	40	18.99	0.58
HC+ WC + *C.dactylon* + AMF + ECCN84	60	17.74	0.46
HC+ WC + *C.dactylon* + AMF + ECCN84	80	16.46	0.40
HC+ WC + *C.dactylon* + AMF + ECCN84	100	14.36	0.29
HC+ WC + *C.dactylon* + AMF + ECCN84	120	13.13	0.15
HC+ WC + *C.dactylon* + AMF + ECCN84	140	10.96	0.12
HC Control	20	10.21	0.03
HC Control	40	10.25	0.02
HC Control	60	10.44	0.02
HC Control	80	10.45	0.02
HC Control	100	10.08	0.01
HC Control	120	9.80	0.02

Table A4.3. Moisture content value standard errors at each point of analysis within treated and control columns. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control. Values in treated columns significantly different to values in the hard coal control column at the corresponding point are indicated in red.

Treatment	**Column Depth (cm)**	**Mean Value**	**Standard Error**
HC+ WC + *C.dactylon*	20	24.73	1.85
HC+ WC + *C.dactylon*	40	9.12	0.59
HC+ WC + *C.dactylon*	60	9.44	0.21
HC+ WC + *C.dactylon*	80	9.76	0.71
HC+ WC + *C.dactylon*	100	10.56	0.49
HC+ WC + *C.dactylon*	120	7	0.69
HC+ WC + *C.dactylon*	140	10.96	1.15
HC+ WC + *C.dactylon* + AMF	20	5.42	0.15
HC+ WC + *C.dactylon* + AMF	40	9.10	0.04
HC+ WC + *C.dactylon* + AMF	60	6.93	0.48
HC+ WC + *C.dactylon* + AMF	80	7.72	0.05
HC+ WC + *C.dactylon* + AMF	100	7.08	0.77
HC+ WC + *C.dactylon* + AMF	120	10.5	1.27
HC+ WC + *C.dactylon* + AMF	140	6.90	0.03
HC+ WC + *C.dactylon* + AMF + ECCN84	20	24.21	0.87
HC+ WC + *C.dactylon* + AMF + ECCN84	40	7.94	0.32
HC+ WC + *C.dactylon* + AMF + ECCN84	60	6.96	0.21
HC+ WC + *C.dactylon* + AMF + ECCN84	80	6.7	0.13
HC+ WC + *C.dactylon* + AMF + ECCN84	100	7.94	0.02
HC+ WC + *C.dactylon* + AMF + ECCN84	120	7.83	0.25
HC+ WC + *C.dactylon* + AMF + ECCN84	140	8.51	0.04
HC Control	20	9.97	0.12
HC Control	40	15.68	0.08
HC Control	60	11.21	0.42
HC Control	80	20.59	0.48
HC Control	100	17.47	1.17
HC Control	120	16.83	0.74

Table A4.4. pH value standard errors at each point of analysis within treated and control columns. Treatments compared; Hard Coal + Weathered Coal + *Cynodon dactylon*, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi, Hard Coal + Weathered Coal + *Cynodon dactylon* + Arbuscular Mycorrhizal Fungi + EBRU Culture Collection Isolate Number 84 and a Hard Coal control. Values in treated columns significantly different to values in the hard coal control column at the corresponding point are indicated in red.

Treatment	**Column Depth (cm)**	**Mean Value**	**Standard Error**
HC+ WC + *C.dactylon*	20	4.10	0.01
HC+ WC + *C.dactylon*	40	4.03	0.01
HC+ WC + *C.dactylon*	60	3.93	0.01
HC+ WC + *C.dactylon*	80	3.69	0.02
HC+ WC + *C.dactylon*	100	3.85	0.01
HC+ WC + *C.dactylon*	120	3.96	0.01
HC+ WC + *C.dactylon*	140	4.02	0.01
HC+ WC + *C.dactylon* + AMF	20	4.12	0.01
HC+ WC + *C.dactylon* + AMF	40	3.97	0.01
HC+ WC + *C.dactylon* + AMF	60	3.83	0.01
HC+ WC + *C.dactylon* + AMF	80	3.68	0.01
HC+ WC + *C.dactylon* + AMF	100	3.74	0.01
HC+ WC + *C.dactylon* + AMF	120	3.77	0.01
HC+ WC + *C.dactylon* + AMF	140	3.80	0.01
HC+ WC + *C.dactylon* + AMF + ECCN84	20	4.50	0.01
HC+ WC + *C.dactylon* + AMF + ECCN84	40	4.22	0.011
HC+ WC + *C.dactylon* + AMF + ECCN84	60	3.98	0.02
HC+ WC + *C.dactylon* + AMF + ECCN84	80	3.77	0.01
HC+ WC + *C.dactylon* + AMF + ECCN84	100	3.73	0.01
HC+ WC + *C.dactylon* + AMF + ECCN84	120	3.74	0.01
HC+ WC + *C.dactylon* + AMF + ECCN84	140	3.64	0.02
HC Control	20	3.64	0.01
HC Control	40	3.61	0.01
HC Control	60	3.46	0.01
HC Control	80	3.56	0.01
HC Control	100	3.56	0.01
HC Control	120	3.69	0.01