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The role of a plant/fungal consortium in the degradation of bituminous hard coal

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Abstract The sporadic growth of *Cynodon dactylon* was observed to occur directly on the surface of hard coal in dumps of the Witbank coal mining area of South Africa with the surface coal being broken down into a humic-like particulate material. Microorganism analysis of plants and rhizosphere material from the dumps revealed the presence of arbuscular mycorrhizal fungi and the coal solubilising fungus, *Neosartorya fischeri*. Studies established to replicate the dump environment revealed increased coal degradation in the form of humic acid production and an increase in small size particles as a result of *Cynodon dactylon* growth in association with arbuscular mycorrhizal fungi and *Neosartorya fischeri*. Results suggest that interactions between *Cynodon dactylon*, arbuscular mycorrhizal fungi, *Neosartorya fischeri* and other coal-degrading rhizosphere fungi could lead to the degradation of hard coal in situ and that the application of these organisms to discard dumps could be a novel method of coal dump rehabilitation.

Keywords Hard coal · Coal discard dumps · Rehabilitation · Biodegradation · Fungi · *Neosartorya fischeri* · Arbuscular mycorrhizal fungi

Introduction

Due to its organic origin and similarities to lignin, it has been hypothesised that coal is susceptible to transformation by living organisms such as bacteria and fungi (Catcheside and Ralph 1998; Hayatsu et al. 1979). The biodegradation of hard coal by fungal species first reported by Fakoussa (1981) and subsequently by Cohen and Gabriele (1982) led to a series of studies that demonstrated the ability of a range of microbial species that are able to catalyse this process (Catcheside and Ralph 1998; Cohen and Gabriele 1982; Cohen et al. 1990; Fakoussa and Hofrichter 1999; Laborda et al. 1999; Ralph and Catcheside 1994). However, most of the studies carried out focussed on the degradation of lignites and low rank coals. Limited success has been reported in the biodegradation of hard coal. Similarly, large-scale industrial applications utilising such microbial systems have been limited.

Igbinigie et al. (2008) reported the effective breakdown of bituminous hard coal on discard coal dumps in the Witbank coal producing region of South Africa that had been colonised by *Cynodon dactylon* over time. Below this grass cover, the top layer of the coal

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had been broken down into a humic-like particulate material. Colonisation of the *C. dactylon* roots by arbuscular mycorrhizal (AM) fungi as well as the presence of a number of fungal species within the rhizosphere was reported. Among this fungal consortium was *Neosartorya fischeri* that was shown to have the ability to degrade hard bituminous coal in pure culture flask and perfusion bed reactor studies. Igbinigie et al. (2008) suggested that the introduction of photosynthates into the rhizosphere zone underpinned a complex interaction between plant, AM and rhizosphere fungi which resulted in the breakdown of coal.

Results obtained by Igbinigie et al. (2008) further enhanced the suggestion that the breakdown of coal observed in the Witbank coal producing area was as a result of complex interactions in the rhizosphere. However, the possible roles of alternative rhizosphere fungi in this process, most notably AM fungi, was not investigated. Further, studies excluded the actual presence of *C. dactylon* plants themselves. This study investigated such possible interactions using bench-scale pot and column trials involving the hard coal substrate and were configured to simulate the upper 1.5 m layer of the coal dump environment where the majority of plant and microbial interaction appeared to occur.

Materials and methods

Column trials

Plastic columns (1.5 m high, 12.7 cm wide and 6.7 cm deep) were constructed and packed with coal to simulate the upper layer of the dump environment. The columns were fitted with a detachable see-through perspex face and filled to the 120 cm mark with discard bituminous hard coal (HC) sourced from the AngloCoal Kroomdraai Mine, Navigation dump site (Witbank, South Africa). *C. dactylon* seed (0.5 g) was planted into each column alongside a number of microbial treatments. For treatments requiring inoculation with AM fungi, 23 g of a mycorrhizal inoculum (64 viable propagules g^{-1}) developed from the original dumpsite by Mycoroot (Pty) Ltd (Grahamstown, South Africa) was added to a column. Mycorrhizal species present within the inoculum were *Glomus clarum*, *Paraglomus occultum*, *Gigaspora gigantea* and *Glomus mossea*. For treatments requiring the addition of *N. fischeri*, 100 ml of a sterile spore suspension (7×10^4 spores ml^{-1}) derived from the washing of Potato Dextrose Agar petri-dish cultures with distilled water was added to a column. Weathered coal (WC) was added to the top 20 cm section of each treated column as an easily degradable amendment providing for the initiation of the process as reported by Igbinigie et al. (2008) and consisted of 25% WC and 75% HC.

Treatments included: (1) hard coal (HC) + weathered coal (WC) + *C. dactylon*; (2) HC + WC + *C. dactylon* + AM fungi; (3) HC + WC + *C. dactylon* + AM fungi + *N. fischeri*; (4) HC control.

The HC control consisted of a column packed to the 120 cm mark with discard bituminous hard coal without the addition of a weathered coal amendement, *C. dactylon*, AM fungi or *N. fischeri*, representing an un-rehabilitated discard coal dump. Two replicates of each treatment were planted and columns were harvested and analysed after 44 weeks. Prior to harvesting, columns were maintained at a set temperature of 25°C with a day/night regime of 16/8 h. Columns were watered daily with 100 ml of water. Column trials were conducted at the Environmental Biotechnology Research Unit, Rhodes University (Grahamstown, South Africa).

Column conditions were examined by the removal of the Perspex cover and sampling was conducted at 20 cm intervals down the column length.

Pot trials

Pots 17 cm deep and 20 cm wide were prepared in a similar way to the columns. The lower two-thirds of the pot was filled with discard bituminous hard coal and the upper third of the pot was modified with a WC coal amendment. *C. dactylon* seed (0.5 g) was distributed evenly into each pot and slightly covered. For treatments requiring AM fungi, 10 g of mycorrhizal inoculum was added to each pot. For treatments requiring inoculation with *N. fischeri*, 50 ml of a spore suspension (5×10^4 spores ml^{-1}) was added to each pot. Treatments replicating those established in the column trials were established with the addition of a treatment consisting of hard coal + weathered coal + *C. dactylon* + *N. fischeri*. No HC control was established.

Nine replicates of each treatment were planted. Pots were maintained at a set temperature of 25°C

with a day/night regime of 16/8 h. Pots were watered daily with 50 ml of water.

Pot trials were conducted at the Environmental Biotechnology Research Unit, Rhodes University (Grahamstown, South Africa) and were primarily utilised to identify the rhizosphere fungal species present within the proposed system.

Humic acids

The production of humic acid in each treated column was measured as an indication of coal breakdown and analysis followed the methods of Magi et al. (1995). Coal samples (10 g) were taken from each sampling point within each column and diluted in 100 ml distilled water. Three 7 ml samples were acidified to below pH 1 by the addition of 150 µl concentrated HCl. Samples were incubated at room temperature for 24 h before being centrifuged at 4,000 rpm for 90 min at 10°C. The supernatant was removed and the pellet re-suspended in 7 ml 0.1 M NaOH. Sodium carbonate (1.05 ml) of 200 g L^{-1} concentration and 0.35 ml of Folin-ciocalteu's phenol reagent was added to each sample and samples were incubated at room temperature for 60 min. The optical density (OD) of each sample was then measured at 750 nm. Humic acid concentrations were measured against a standard curve ($y = 0.003x$, $R^2 = 0.93$).

Size fractionation

Changes in coal particle size were measured as an indication of the degradation effects of the various treatments. Whole substrate sections (0–20 cm from the column top) of separate treated coal columns were removed (WC material). Samples were oven dried at 60°C overnight and dried samples were fractionated through a nest of sieves (mesh sizes of 8 mm, 2 mm, 1 mm and 500 µm). The fraction collected from each sieve was weighed.

Scanning electron microscopy

Fungal attack on the coal surface was observed using scanning electron microscopy (SEM). Coal samples from differentially treated columns were fixed, dehydrated, critically dried, and gold-coated according to the methods of Cross and Pinchuck (2001). Prepared samples were examined with a VEGA LMU (VEGA© Tescan) Scanning Electron Microscope at Rhodes University, Grahamstown.

Mycorrhizal colonisation

Arbuscular mycorrhizal colonisation of plant roots was observed using light microscopy to determine whether colonisation was present through the length of the column. Composite samples (0.5 g in total) of young root tip sections (5 mm) were harvested from the top (20 cm), middle (80 cm) and bottom (140 cm) column sections. Roots were carefully washed with water and then cleared, bleached, acidified and stained with Trypan Blue according to the methods of Smith and Dickson (1997). Root sections were then de-stained for 24 h prior to examination under a Nikon YS100 compound microscope. The presence of colonisation was recorded.

Identification of rhizosphere fungi

Ribosomal deoxyribonucleic acid (rDNA) analysis was utilised for the characterisation of microorganisms present in the rhizosphere as well as the mycorrhizal inoculum developed from the Navigation dump site. Total genomic DNA extractions following the methods of Bond et al. (2000) were carried out on 1 g samples of rhizosphere coal from across all treatments as well as 1 g samples of mycorrhizal inoculum prior to Polymerase Chain Reaction (PCR) amplification of specific rDNA sequences.

Internally transcribed spacer (ITS) regions were utilised to identify the rhizosphere fungi present within each treatment. PCR amplification of fungal ITS regions was carried out in 25 µl reactions consisting of 2 µl extracted genomic DNA, 2 µl ITS1-F Primer (5 mM), 2 µl ITS4 Primer (5 mM), 1 µl dNTPs (10 mM), 1 µl BSA (50 mg ml^{-1}), 2 µl 25% DMSO, 2.5 µl Magnesium-free Buffer, 1.5 µl $MgCl_2$ (25 mM), 0.2 µl Taq polymerase and 10.8 µl sterile distilled water using a GeneAmp 9700 PCR Thermocycler. Reaction parameters utilised replicated those used by Larena et al. (1999). The primer set of ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) utilised primarily amplified both ITS regions of fungal rDNA as well as the 5.8S subunit separating them (Martin and Rygiewicz 2005).

The primer set of AM1 (Helgason et al. 1998) and NS31 (Simon et al. 1992) was used to confirm

identification of mycorrhizal species present within the inoculum used in trials. This primer set amplifies a portion of AM fungal small sub-unit rRNA gene (Douhan et al. 2005). Similarly, 25 µl reactions were amplified as described above, however, 10 mM concentrations of primers were used in each reaction. PCR parameters involved an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 48°C for 1 min and an elongation at 72°C for 8.5 min. A final elongation step at 72°C for 8.5 min completed the reaction.

Extension products were cloned into the pGEM T-Easy Vector System (Promega) and subsequent transformation and blue/white selection on Luria/Ampicillin/Xgal/IPTG agar plates was conducted. Five clones (A–D) from each coal treatment were selected for sequencing. Ten clones were selected for sequencing from extension products generated from the mycorrhizal inoculum. Clones were sequenced using an ABI Prism 3100 Genetic Analyser and organism identities derived using the Basic Local Alignment Search Tool (BLAST) available online from the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Results of over 95% sequence homology were selected.

Sequences were aligned using the CLUSTAL X multiple sequence alignment program and phylogenetic trees were generated using Treeview. Phylogentic trees were rooted with the 16S ribosomal sequences of the archaeal strain *Pyrococcus horikoshii* OT3. Phylogenetic tree graphics were finalised using CanvasX from ACD systems.

Root exudates

Organic acids exuded by mycorrhizal plants which may aid in the oxidation of coal were identified using Gas Chromatography-Mass Spectrometry (GC-MS). Both mycorrhizal inoculated and non-mycorrhizal inoculated plants were hydroponically grown in sterilised plastic PVC pots (20 cm long and 4.5 cm in diameter) for a period of 12 months.

Pipes were four-fifths filled with an inert clay carrier after which 2 g of *C. dactylon* seed and 7.5 g mycorrhizal inoculum, where required, were applied. Five replicates were established for mycorrhizal and non-mycorrhizal treatments. Pipes were maintained at a min/max temperature of 18/25°C and were watered once daily with 30 ml ultra-violet sterilised water and once weekly with 20 ml low P Long Ashton's Nutreint Solution (Hewitt 1952).

Sterile deionised water was drained through the carrier in which mycorrhizal and non-mycorrhizal plants were grown for a period of 10 h and collected in a 250 ml beaker. Root exudation patterns would have continued during this time as the soluble organic acids exist as anions in soil and can be collected in water (George et al. 2002; Szmigielska et al. 1996). Water samples (50 ml) from the two treatments were collected and centrifuged at 5,000 rpm for 5 min at 4°C in an Eppendorf 5810 R centrifuge to pellet any debris and the supernatant collected. A sample (300 µl) was collected and potential organic acids within root exudates derivitised and assessed by the methods described by Villas-Bôas et al. (2003).

Statistical analysis

Data is represented as mean values of three replicates with standard errors reported. Statistical analyses of results were undertaken using STATISTICA (version 7.1). The Fisher LSD one-way analysis of variance (ANOVA) test was used to compare results with a 95% degree of confidence where the level of statistical significance was accepted at $P < 0.05$ (Zar 1998).

Results

Humic acid

The breakdown of the coal in the column trials showed an increasing production of humic acid across the treatment series of *C. dactylon* + WC; *C. dactylon* + WC + AM fungi and *C. dactylon* + WC + AM fungi + *N. fischeri* compared to the HC control (Fig. 1). At a depth of 20 cm into each column, significant differences ($P < 0.05$) in humic acid concentrations between treatments and the HC control were observed with concentrations of 116.7 ppm (*C. dactylon* + WC + AM fungi + *N. fischeri*), 97.5 ppm (*C. dactylon* + WC + AM fungi) and 56.7 ppm (*C. dactylon* + WC) recorded in treated columns. This was compared to the 18.2 ppm humic acid concentration within the HC control column. Significant differences in humic acid concentrations remained between coal from the various treatments and HC control down to the 60 cm depth of each

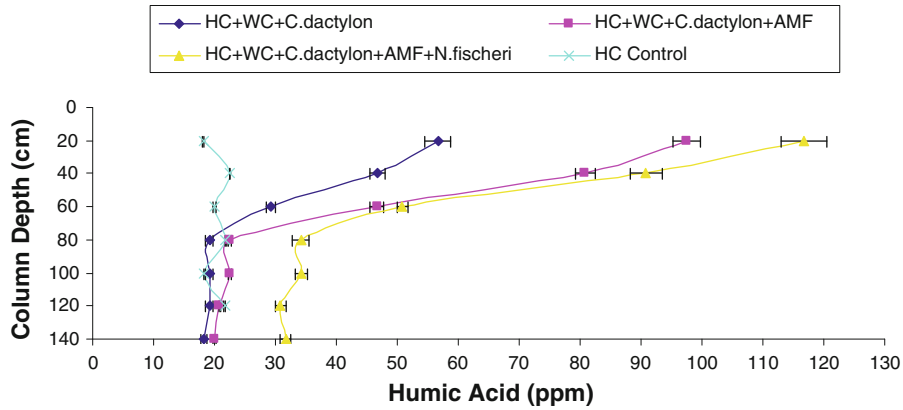

Fig. 1 Depth profile illustrating humic acid levels at various depths within treated and control columns. Treatments compared; hard coal (HC) + weathered coal (WC) + *Cynodon dactylon*, hard coal + weathered coal + *Cynodon dactylon* + arbuscular mycorrhizal fungi (AMF), hard coal + weathered coal + *Cynodon dactylon* + arbuscular mycorrhizal fungi + *Neosartorya fischeri* and a hard coal control. Horizontal bars represent standard error

column. Below this level, the differences observed between treated and HC columns were not significant (Fig. 1).

Size fractionation

Size fractionation of column WC amendments after 44 weeks showed an accelerated breakdown of the coal substrate reflected as an increase in smaller particle size fractions in the various treatments compared to the HC control (Fig. 2). In the largest (8 mm) size fraction, across the series of treatments, an average 64.2% particle size reduction was observed in comparison to control substrate material (untreated WC amendment). Slight variation was seen between the various treatments and control material in the 2 mm size fractions collected. In the 1 mm, 500 µm and <500 µm particle size fractions collected, average percentage increases of 43.4, 28.9 and 34.9%, respectively, were observed over the 44-week

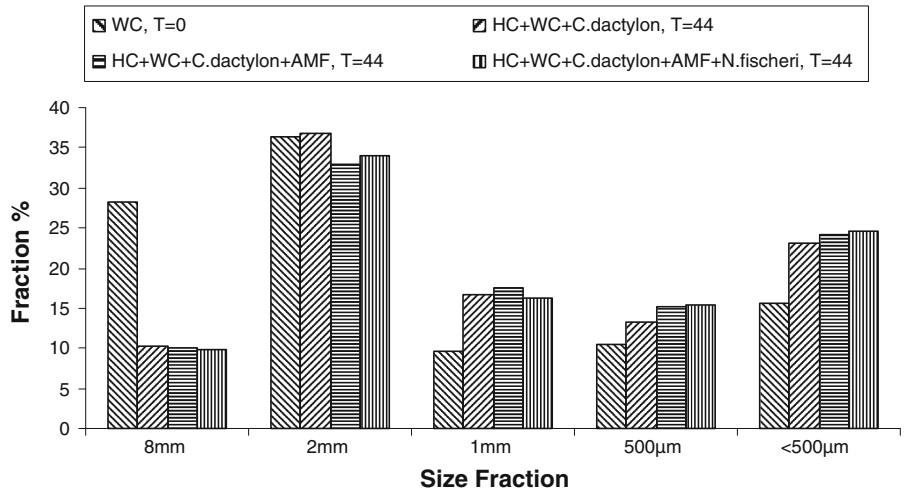

Fig. 2 Size fractionation percentages of coal substrate in column studies comparing various treatments over a 44-week period. Weathered coal material at $T = 0$ is compared to material from the column treatments hard coal (HC) + weathered coal (WC) + *Cynodon dactylon*, hard coal + weathered coal + *Cynodon dactylon* + arbuscular mycorrhizal fungi (AMF), hard coal + weathered coal + *Cynodon dactylon* + arbuscular mycorrhizal fungi + *Neosartorya fischeri*

study for the various treatments in comparison to control substrate material. Again, little variation was observed between the various treatments although a slight trend is apparent across the treatment series as was noted in the humic acid production results.

Scanning electron microscopy

Scanning electron microscopy showed extensive fungal colonisation of coal particles. This was most developed in columns with *C. dactylon* growing in combination with the AM fungal and *N. fischeri* inoculum. Proliferation of hyphae into cracks and fissures developing on the coal particle surface was further observed (Fig. 3) and a flaking of the coal surface appeared to occur and lead to breakdown into the smaller particle sizes observed. No hyphal proliferation was observed in the HC control treatments.

Mycorrhizal colonisation

Light microscopy analysis of root samples revealed the presence of mycorrhizal colonisation in all samples obtained from treatments inoculated with AM fungal inoculum indicating that roots down the length of the column were successfully colonised. The highest colonisation percentages were observed in root samples obtained from the 0–20 cm sections of the columns coinciding with the highest amounts of root biomass visually observed (data not shown). Within this column section, the colonization percentage within newly formed root tip sections of the *C. dactylon* + WC + AM fungi + *N. fischeri* treated column (11.75%) was significantly higher than percentages recorded within remaining treatments. Colonization percentages remained highest in this treatment down the length of the column.

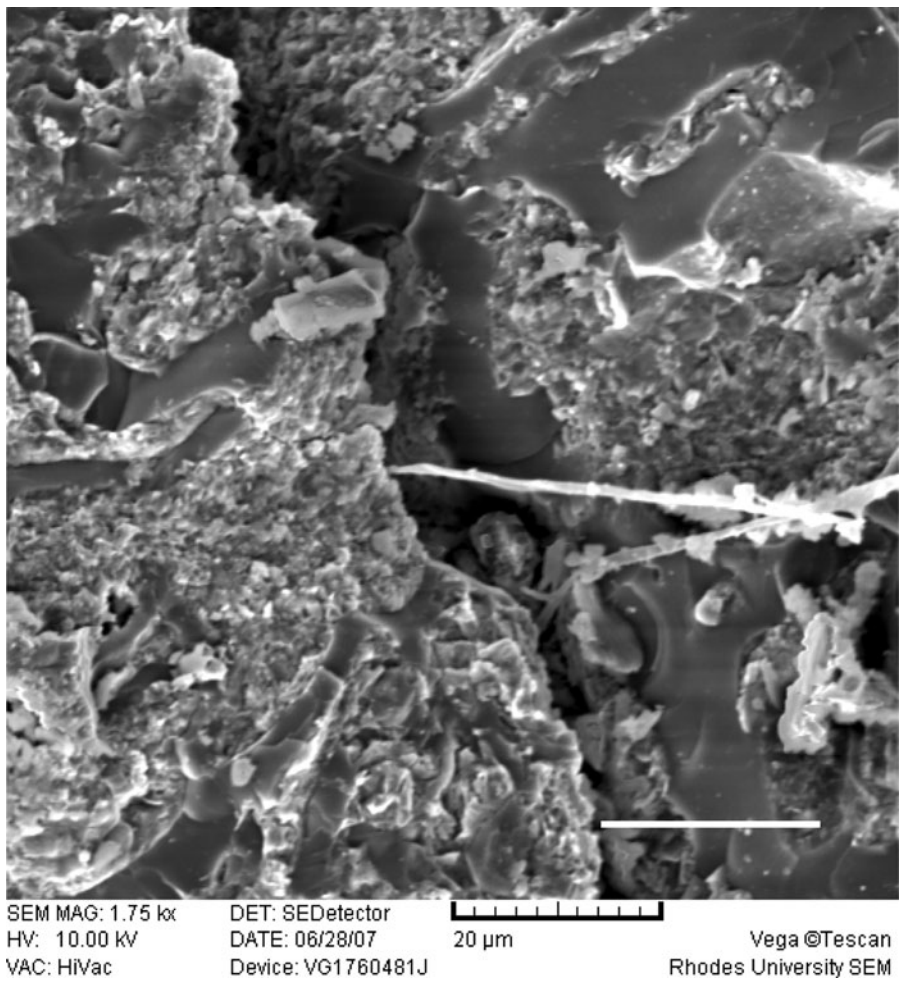

Fig. 3 Scanning electron micrograph showing hyphal penetration into a coal particle where surface shearing has occurred. The coal particle was obtained from a column treated with *Cynodon dactylon*, arbuscular mycorrhizal fungi and *Neosartorya fischeri*

Identification of rhizosphere fungi

Organisms present in the rhizosphere of each treatment were identified using total genomic DNA extractions and molecular characterisation. For treatments inoculated with AM fungi and/or *N. fischeri*, *N. fischeri* was genetically confirmed from the treatment consisting of *C. dactylon* + WCl + AM fungi + *N. fischeri* confirming its presence in the rhizosphere after 44 weeks (Fig. 4). *N. fischeri* was not genetically traced from treatments involving its sole inoculation alongside *C. dactylon* or treatments where the isolate was not added to columns. Specific AM fungal species were not genetically traced from the rhizosphere of any of the treatments established. A high percentage of the alternative species genetically identified from the rhizosphere of the range of treatments belonged to the genera *Trichoderma* and *Penicillium*. Other prominent organisms identified across the series of treatments were species belonging to the *Rhodosporidium* and *Mycosphaerella* genera.

Specific AM fungal species present within the mycorrhizal inoculum utilized in treatments were identified using total genomic DNA extractions and molecular characterisation and confirmed to be *G. clarum*, *Pa. occultum*, *Gi. gigantea* and *G. mossea* (Fig. 5).

Root exudates

Chromatographic analysis of root exudates from mycorrhizal and non-mycorrhizal *C. dactylon* plants revealed a heavy presence of chlorinated and aromatic compounds. Organic acids were not detected in the root exudates of non-mycorrhizal plants (data not shown), however, the methylated form of fumaric acid, 2-butenedioic acid (E)-, dimethyl ester (asterisk denoted) was identified within the root exudates of mycorrhizal plants (Supplementary Fig. S1; Table 1).

Discussion

The aim of this study was to investigate the possible role played by rhizosphere fungi in association with *C. dactylon* in the degradation of hard coal. Experimental trials under controlled lab conditions were conducted to simulate the coal dump environment where the sporadic growth of *C. dactylon* was observed to occur directly on the surface of hard coal as reported by Igbinigie et al. (2008). The coal in the upper layers of these dumps was observed to be broken down into a humic-like particulate material. Igbinigie et al. (2008) proposed that an association between the plant and rhizosphere microorganisms may be playing a role in the degradation of the coal but did not have rigorous data to confirm this observation. Therefore, to comment on the biology of the system and determine whether it was biologically driven, a series of biological treatments were established to interrogate this hypothesis.

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 (Catcheside and Ralph 1998; Machović et al. 2000; Senesi and Milano 1994). In this study, the production of humic acid was interpreted as a reflection of coal breakdown. Results obtained demonstrated that *C. dactylon* by itself was able to degrade coal, but in combination with either AM fungi and/or *N. fischeri*, this degradation was enhanced (Fig. 1).

The most commonly sequenced region utilised in the identification of fungi is the internal transcribed spacer region of the nuclear ribosomal repeat unit (Nilsson et al. 2010). Though not completely trouble-free, with the 18S rRNA gene often solely sequenced for higher specificity, the ITS region was targeted in this initial study to provide a better indication of fungal groups and genera present within the system (Feliner and Rosselló 2007; Woo et al. 2010). Further studies should also look into the utilization of denaturing gradient gel electrophoresis (DGGE) to eliminate potential errors brought about by PCR, cloning and sequencing (Middleton et al. 2004).

Limited numbers of genetic sequences were derived from this study, however, results provided a significant indication of fungi present within the dump environment. Genetic analysis recovered *N. fischeri* from the rhizosphere of the treatment consisting of *C. dactylon* + AM fungi + *N. fischeri*, an isolate Igbinigie et al. (2008) showed to posses coal-solubilising ability (Fig. 4). Arbuscular mycorrhizal fungal species were not directly identified from the rhizosphere of the series of treatments to which inoculum was added. However, microscopic analysis of plant roots from all the treatments involving inoculation with arbuscular

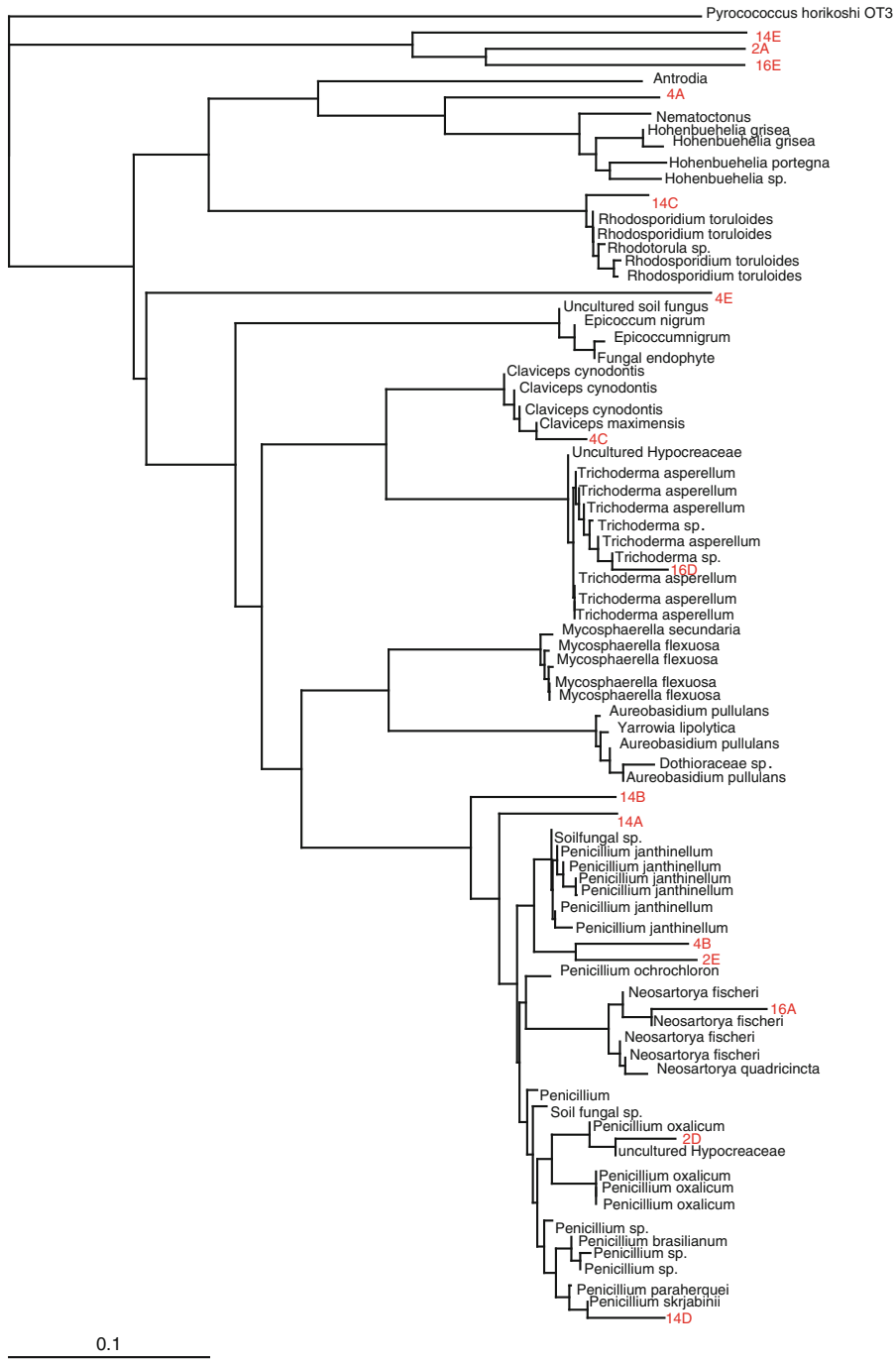

Fig. 4 Rooted phylogram illustrating fungal species of rhizosphere organisms isolated from pots that underwent various treatments. Numerals are reference to treatments from which species were isolated. Clone number *2 C. dactylon* + WC, clone number *4 C. dactylon* + weathered coal + arbuscular mycorrhizal fungi, clone number *14 C. dactylon* + weathered coal + *Neosartorya fischeri*, clone number *16 C. dactylon* + weathered coal + arbuscular mycorrhizal fungi + *Neosartorya fischeri*

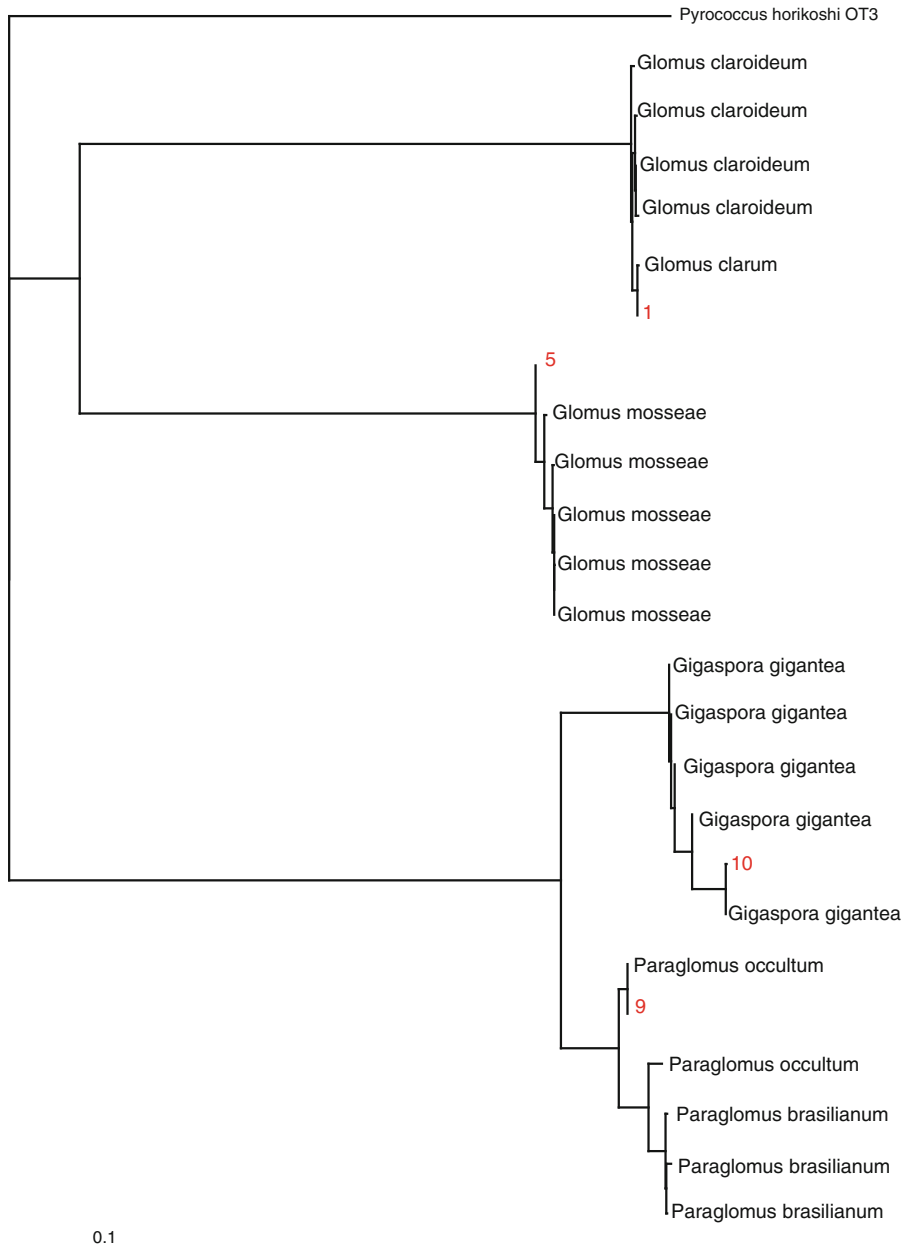

Fig. 5 Rooted phylogram illustrating the species of arbuscular mycorrhizal fungi present in the inoculum utilised in treated trials. *Numerals* represent the clone number from which molecular characterisation results were obtained

mycorrhizal fungi confirmed their presence in newly formed root material throughout the length of the column, be it at relatively low colonisation percentages. Although the fungal ITS primers used in this study would have amplified sections of the mycorrhizal genome, the number of clones selected per treatment was limited and amplified mycorrhizal genomic segments may have been overlooked. Extraradical AM hyphal growth may have further been limited by saprotrophic fungal development within the system. Similarly, such development could have also limited intraradical colonisation, as was observed. Both rhizosphere-dwelling and root-colonising fungi, pathogenic and non-pathogenic, have the ability to

Table 1 Retention times of compounds present within root exudates collected from mycorrhizal plants. Times relate to compound peaks detected from GC-MS analysis (Supplementary Fig. S1)

Peak number	Retention time (min)	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

reduce mycorrhizal colonisation in plants (Cordier et al. 1998; Muller 2003; Yao et al. 2002).

Together with mechanical weathering, via the exudation of various organic compounds, mycorrhizal fungi possess the ability to weather such material as rock, with the main aim being the derivitisation of primary minerals (Landeweert et al. 2001; van Breemen et al. 2000). Arbuscular mycorrhizal fungi have also previously been successfully utilised to phytoremediate/rehabilitate and revegetate coal mines with the enhancement of plant tolerance to heavy metals and stress proposed to be the key factor in this (Ganesan et al. 1991; Ning and Cumming 2001; Cumming and Ning 2003; Stahl et al. 1988). Also recovered across the series of treatments were a number of other fungi from genera (predominantly *Trichoderma*, *Penicillium*, *Epicoccum*, *Claviceps*, *Metarhizium*, and *Cladosporium*) often found on coal dumps and known to have coal degrading capabilities (Achi 1994; Hölker et al. 2002; Hölker and Höfer 2002; Pokorný et al. 2005). Results suggest that a combination of AM fungi in association with the plant and such rhizosphere organisms resulted in the highest amounts of coal degradation. Size fractionation results mirrored humic acid production results with an increased weathering function observed in the presence of *C. dactylon*, AM fungi and *N. fischeri* (Fig. 2). Inoculation with these organisms resulted in a clear increase in small size particles of coal reflecting degradation. Colonization of the coal surface and hyphal fractionation of the coal structure in treatments inoculated with *C. dactylon*, *C. dactylon* + AM fungi and *C. dactylon* + AM fungi + *N. fischeri* was also observed in comparison to the lack of colonization seen in hard coal control treatments. This observation suggested that *C. dactylon* plays a key role in the stimulation of the rhizosphere population, which does not occur in the HC controls.

Levels of mycorrhizal colonization recorded within roots sampled from the columns were relatively low most likely due to the nature of the coal substrate. Further, the non-destructive method of root sampling down column lengths did limit root amount material available for analysis could have also limited the detection of mycorrhizal fungi within roots. However, similar analysis from roots sampled from established pots trials showing similar levels of colonisation more importantly showed substantial plant growth improvement (data not shown) in the presence of AM fungi, as well as *N. fischeri* (Mukasa Mugerwa 2008). Such results indicated the potential use of AM fungi as well as *N. fischeri* in the bioremediation of hard coal.

Literature has reported that organic acids produced by mycorrhizal fungi could play a significant role in

their oxidation of rock-like material, a phenomenon readily observed (Jongmans et al. 1997; Van Breemen et al. 2000). Organic acid production by mycorrhizal fungi primarily aids in the solubilisation of rhizosphere P with fumaric acid shown to be a dicarboxylic acid also exuded by mycorrhizal fungi (Ouahmane et al. 2007). The fact that an organic acid was identified in the exudates of mycorrhizal plants and not in the exudates of non-mycorrhizal plants strongly suggests that it was produced by the mycorrhizal association. At least, concentrations of organic acids were enhanced by the mycorrhizal fungi as opposed to production by the plant alone. As an organic acid, Fumaric acid would have aided in the oxidation of coal.

While substantial future work is required to establish an accurate description of the processes involved in the degradation of coal observed at the Navigation dump site, results make it possible to formulate a preliminary descriptive model of the system. The study primarily focussed on the roles of specific groups of fungi in this process, however, it was noted that bacteria are also key organisms found in any rhizosphere environment that readily influence soil carbon cycles and function as well as having the ability to solubilize hard coal (Fakoussa 1981; Lavelle and Spain 2001). Therefore, under natural conditions, bacteria present within the investigated system could significantly contribute towards the processes observed and complete microbial consortiums should be investigated in further studies.

Studies have demonstrated that plant exudations in the form of carbohydrates, carboxylic acids and amino acids can directly and indirectly enhance microbial growth in the rhizosphere (Grayston et al. 1998). Results suggest that photosynthate release from the plant stimulates the growth and activity of rhizosphere organisms including those with coal-degrading capability. Available carbon concentrations into the rhizosphere could subsequently be enhanced by arbuscular mycorrhizal fungi in the form of organic acid exudation (Supplementary Fig. S1). Further inoculation with known coal-degrading microorganisms would thus increase the process of coal degradation.

Experimental results confirmed the speculation by Igbinigie et al. (2008) that the degradation of coal was linked to a consortium of microorganisms. Traits possessed by arbuscular mycorrhizal fungi alongside the ability of rhizosphere organisms such as *N. fischeri*

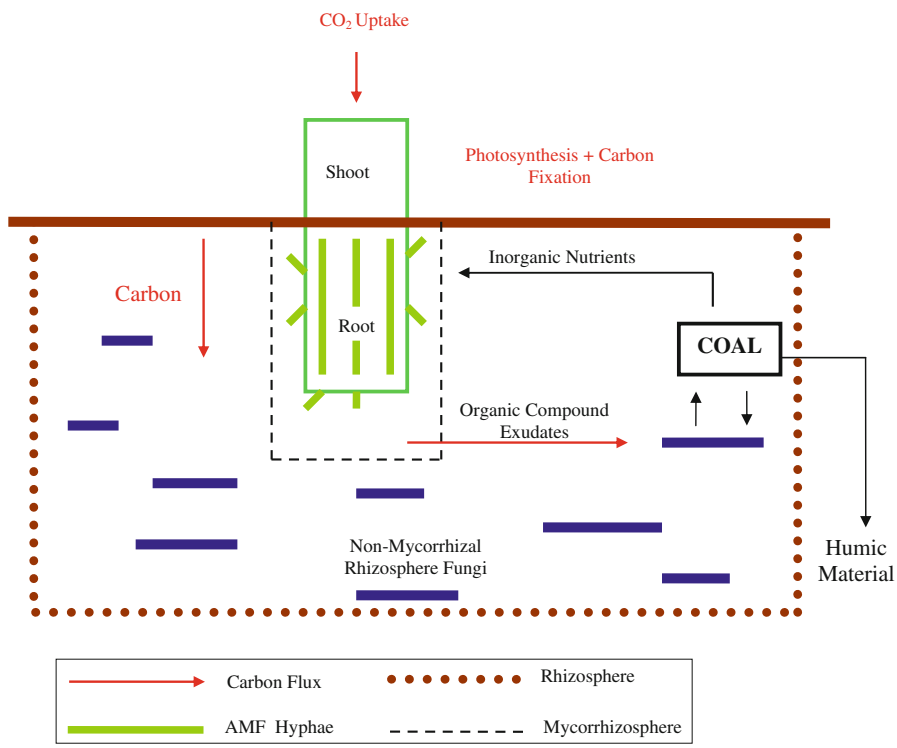

Fig. 6 Schematic diagram illustrating the possible association between mycorrhizal *Cynodon dactylon* and rhizosphere organisms that leads to the degradation of coal in situ. Carbon transport is indicated by *red arrows*

to solubilise coal would have played key roles in the establishment of *C. dactylon* on discard coal dumps. An enhanced carbon supply to the rhizosphere due to plant and mycorrhizal fungal exudations could enhance the presence of rhizosphere coal-solubilising fungi. Solubilisation of the coal substrate would not only yield humic-like material but also make available nutrients directly to the plant itself with both results enhancing plant establishment on coal (Fig. 6).

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