CHAPTER THREE

MOBILISATION OF POTASSIUM AND PHOSPHORUS FROM IRON ORE BY ECTOMYCORRHIZAL FUNGI

Abstract

Mutualistic roles of ectomycorrhizal (ECM) fungi have been linked to their ability to produce organic acids that aid in the dissolution of insoluble minerals in the rhizosphere. This ability of ECM fungi was utilised to investigate their potential participation in the mobilisation of nutrients such as phosphorus (P) and potassium (K) from a typical insoluble ore - iron ore. *In vitro* pure cultures of four different ECM fungi; *Pisolithus tinctorius* (PT), *Paxillus involutus* (PI), *Phialocephala fortini* (PFR) and *Suillus tomentosus* (ST), were screened for their ability to mobilise P and K from two types of non-exportable Sishen iron ore. When present in iron ore, these elements are deleterious and reduce the commercial values of the ore. Experiment was set up with different treatments that included two ore types (KGT and SK) and five particle sizes of each ore type. Results indicated the potential of the four fungi to mobilise P and K from the two iron ore types though at different levels. Ore type, particle size, organic acid production and attachment of the fungi to the iron ore were all found to play important roles in the mobilisation of nutrients from these ores.

3.1 Introduction

Different kinds of interactions exist between microorganisms and minerals in the environment (Erlich, 1997; Gadd, 1999). These interactions have been extensively studied in the mining industries to produce technologies that are beneficial such as mineralisation, bioremediation and biohydrometallurgy (Ehrlich, 1997; Gadd, 1999; Rawlings, 2002). Though many studies have been conducted in most of these areas, there are still some gaps in the understanding of the full potential and applicability of microorganisms such as ectomycorrhizal (ECM) fungi.

On the basis of available literature, Rinaldi *et al.* (2008) conservatively estimated the number of ECM fungi to be 7750 but went further to state that the actual number could be between 20000 and 25000, based on known and unknown species of this group of fungi. These fungi can penetrate hard mineral materials for the purpose of nutrient absorption (van Breemen *et al.*, 2000; Smith and Read, 2008). Their primary role is to search for and absorb nutrients under severe soil conditions on behalf of their host plants (Smith and Read, 1997). Both ecological and weathering roles of ECM fungi have been investigated and linked to their ability to produce metabolites such as organic acids (van Breemen *et al.*, 2000). These acids are low-molecular weight compounds and confirmed to have great potential in the solubilisation of complex or hard mineral materials (van Breemen *et al.*, 2000; Paris *et al.*, 1995; Gadd, 1999). These acids are naturally produced by different types of microorganisms in the environment but production seems to depend on the environmental conditions (Burgstaller and Schinner, 1993; Erlich, 1997). For instance, ECM fungi exude organic acids in the rhizosphere in order to initiate a chain of chemical reactions that lead to the breaking down of hard mineral materials. Shortage of base cations in the growth medium and the presence of some minerals in the growth medium can increase the production of organic acid by ECM fungi (van Scholl *et al.*, 2006a). These attributes are the theoretical base for the consideration of ECM fungi as

potential candidates for mobilisation of nutrients from some mineral ores such as the low-grade iron ore.

In South Africa, there is a huge deposit of iron ore that is hosted by the Palaeo-Proterozoic Transvaal Supergroup in the Northern Cape Province of the country; it is currently being exploited by Sishen mine (Carney and Mienie, 2003). The Transvaal Supergroup consists of different subgroups such as Campbell rand, Asbestos Hills and Gamagaga subgroups. High grades iron ore are usually associated with the Asbestos Hills subgroup, while the medium and lower grade iron ore are found in the Campbell Rand and Gamagara subgroups respectively (Carney and Mienie, 2003). The low grade iron ore of Sishen mine has high K (>0.24%) and P (>0.03%) contents that diminishes their market value and could render them non-exportable because of their negative impact on the smelting process in the blast furnace (Parks *et al*., 1990; Yusfin *et al*., 1999; Williams and Cloete, 2008; Delvasto *et al*., 2009).

Most studies in this area have adopted bacteria to clean up such low grade ores especially in biohydrometallurgy of sulfide minerals (Jain and Sharma, 2004; Rawlings, 2005). However, in the leaching of non-sulfidic minerals such as silicate, carbonate and oxide minerals that cannot be directly attacked by sulfur-oxidising bacteria, fungi are the more suitable candidates (Burgstaller and Schinner, 1993; Jain and Sharma, 2004; Rawlings, 2005; Williams and Cloete, 2008). In the leaching of non-sulfide ores, only two fungal types; *Aspergillus* spp. and *Penicillium* spp., have received considerable attention (Burgstaller and Schinner, 1993). In the study conducted on *Aspergillus* and *Penicillium* by Valix *et al*. (2001) it was discovered that a combination of both microbial activities and production of organic acids produced effective leaching of cobalt and nickel. In another study, Castro *et al*. (2000) confirmed the better potential of *A. niger* compared to bacteria (*Bacillus* and *Pseudomonas*) in the leaching of zinc and nickel. *Aspergillus niger* HNA-1 isolated from the surface of iron ore samples was also used by Delvasto *et al*. (2005) for the desphosphorisation of iron ore, while Williams (2008) was able to use metabolites produced by *Aspergillus* sp. for the reduction of P and K content of iron ore.

Despite the remarkable progress that has been made in the use of *Aspergillus* and *Penicillium* spp. for biohydrometallurgical processes, the health implication of their aerosols and the mycotoxins they produce to both human health and agricultural products are of serious concern (Desjardins and Hohn, 1997; Gorny, 2004) . The focus on the use of fungi in biohydrometallurgy should therefore extend beyond their leaching potentials, but should also include their immediate and long-term impact on the environment. Having established the positive effects of ECM fungi in the areas of weathering and nutrient cycling (Smith and Read, 2008), it is interesting to investigate the ability of these fungi in the mobilisation of P and K from a typical mineral ore - Sishen iron ore. The objectives of this study were therefore to investigate the possible biobeneficiation potentials of four ECM fungi and establish the link between the production of organic acids, particle size, ore type and mobilisation of nutrients from ores by ECM fungi.

3.2 Materials and Methods

3.2.1 Origin of fungal isolates and iron ore preparation

Four different isolates were used in this study. These included *Pisolithus tinctorius* (PT)- # PT 7 (Plant Health Care Inc., Pittsburgh, USA), *Paxillus involutus* (PI) - NOF 2340 (Canada), *Phialocephala fortini* (PFR)- PB6B (South Africa) and *Suillus tomentosus* (ST) - UAMH 6252 (Canada).

Two different types of iron ore samples were supplied by Kumba Iron Ore, Ltd. and were originally characterised by the company as KGT (conglomerates) and SK (shale). Particle size effects were also investigated by using different particle sizes of the iron for the experiment. The ore materials (original size of 120 mm to 450 mm - as supplied by the company) were milled and sieved into different particle sizes of <3.36 mm to >1.68 mm, <1.68 mm to >0.84 mm, <0.84 mm to >0.21 mm, <0.21 mm to >0.1 mm, and <0.1 mm. Henceforth, these would be referred to as particle sizes A, B, C, D and E, respectively. Pretreatments of the iron ore samples are as stated in chapter two (section 2.2.2).

3.2.2 Media preparation

Cultures of the ECM fungi were prepared and maintained on Modified Melin Norkrans (MMN) medium (Marx, 1969). Liquid MMN medium was used for this experiment with the source of K omitted and that of magnessium halved. Quantity of P was also reduced because of the omission of KH_2PO_4. The final concentration of the culture medium contained: Malt extract (3 g/l), Glucose (10 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), $MgSO_4.7H_2O$ (0.075 g/l), $CaCl_2.H_2O$ (0.067 g/l), NaCl (0.025 g/l), $FeCl_3.6H_2O$ (0.001 mg/l) and thiamine (100 µg/l). Each of the 100 ml flasks used contained 2.4 g of iron ore per 40 ml of the adjusted concentration of MMN medium while the controls had no iron ore but complete composition of the MMN with 0.5g/l (0.02g/40 ml) of KH_2PO_4 and 0.15g/l (0.006g/40 ml) of $MgSO_4.7H_2O$. Another control was set up to test the media effects

using only iron ore of different particle sizes with the MMN. There were four replicates for each treatment. The flasks used for the experiment were incubated at 28ºC for 28 d and shaken at 100 rev/min.

3.2.3 Organic acid analyses by High Performance Liquid Chromatography (HPLC)

At the end of the experiment, the concentration of the organic acids secreted by the fungi in the growth media was assessed. The growth medium was passed through filter paper (0.45 µm) to separate the fungi and iron ore from the medium of growth. Collected media were homogenised by vortexing, then centrifuged for 180 s at 16060 rpm and the supernatant frozen at –40^{o}C prior to analysis by HPLC.

A volume of 50 µl of the sample was used for HPLC analysis. The chromatographic conditions were a modification of the separation method described in Schneider *et al*. (1987). The HPLC analytic system was controlled by WATERS Empower software (WATERS, Milford, MA, USA) and was composed of a Model 515 pump and a Model 717 ^{plus} autosampler, and a Model 2487 dual absorbance detector. Organic acids were separated on a Bio-Rad HPX-87H column (Bio-Rad, Hercules, Ca, USA) eluted isocratically at 40 °C at a flow rate of 0.6 mL min^{-1} with 0.008 N sulfuric acid and detected on a dual absorbance detector set at 210 nm (Waters, Model 2487). Peak identity and organic acid quantity were determined by comparison with standards. The organic acid standard included oxalic acid, citric acid, malonic acid, maleic acid, lactic acid, acetic acid and formic acid that were well separated under the described chromatographic conditions.

3.2.4 Chemical analyses for nutrients absorbed by the ECM fungi

Based on the result from the mobilisation of P and K from the iron ore samples, further processing of the dried fungal mycelia (65 ^{o}C) and the remaining growth medium were

carried out to analyse the nutrients absorbed by the ECM fungi. This assessment was only done for particle size E treatment. For the digestion of the dried ECM mycelia, samples were dried in the oven at 70 °C, ground in a Wiley mill and passed through a 2-mm sieve screen. A 0.5 g quantity of the each sample was then digested in a sulfuric acid and hydrogen peroxide mix (Parkinson and Allen, 1975). Determinations of P and K were made by Induction Coupled Plasma Optical Emission Spectrometer ICP-OES Optima 4300 DV (Perkin Elmer, Waltham, MA, USA).

3.2.5 Microscopy

Scanning electron microscopy (SEM) was used to view the physical interaction of the ECM fungi with the ore samples. There was random selection of samples from replicates of different treatments for microscopic analysis. The samples were fixed with a solution that contained 2.5% glutaraldehyde, 4% paraformaldehyde in sodium cacodylate buffer, 0.1 M pH 7.3, for 24 h. Fixation was followed by washing with cacodylate buffer three times for 10 min. Post-fixation was carried out with osmium tetroxide (1% in cacodylate buffer), for 90 min, followed by another round of washing with cacodylate buffer, three times for 10 min. The samples were then dehydrated at different alcohol concentrations (50, 70, 95 and 100%) for 10 min each. The dehydrated samples were then soaked in 100% alcohol for an initial period of 40 min and then for another 10 min. This stage was followed by soaking the samples in hexaméthyldisilazan two times for 30 min. Finally, specimens were then air-dried and later coated with gold/palladium. They were then assembled for observation under the microscope at 30 kV on a JEOL 6360LV scanning electron microscope (Tokyo, Japan).

3.2.6 Elemental analyses

X-ray fluorescence (XRF) analyses of iron ore was used to detect K, P and total Fe of the ore samples at the beginning and the end of the experiment. The iron ore samples were collected from the flasks after the treatment. The samples were dried at 110 ^{o}C. Tungsten

carbide milling vessel was used to pulverize the Iron ore samples to a particle size of <75 µm. This was followed by the determination of Lost of Ignition (LOI) which involved roasting of the milled samples at 1000 ºC. Samples (1 g) of the milled iron ore were added to 6 g $Li_2B_4O_7$ and fused into glass beads. Minor elemental analysis was carried out from the powder briquette made from the remaining part of the sample while the major elemental analyses were performed on the fused bead using an ARL 9400XP+ spectrometer at University of Pretoria.

3.2.7 Statistical analyses and experimental design

The statistical analyses were done using SAS software, version 9.2 (SAS Institute 2008, Cary, NC, USA). There were three fixed factors for these analyses and these include fungal isolate (PFR, PI, ST, and PT), iron type (KGT, SK) and particle size (A, B, C, D, E). In addition to these 40 (4X2X5) combination of treatments, there was one control for each fungal isolate consisting of four replicates of isolates PT, PI, PFR and ST and complete MMN medium without iron ore to give a total of 44 treatments. Each of these treatments was replicated four times to give a total of 176 observations.

Dependent variables were in three groups including aqueous P (P_{aq}) and K (K_{aq}) analysed from growth medium as well as P and K from the dried mycelia (P_{dw} and K_{dw}) of the fungi making up the first group while the second group included percentage K and P loss by the iron ore (K_{Loss}, P_{Loss}). K_{Loss} and P_{Loss} were calculated by subtracting the final value of K or P from the initial value prior to the treatments. The total was divided by the initial value of K or P and multiplied by 100. The third group includes organic acids released into the growth medium by the fungi. These were oxalic, citric, maliec, malonic, lactic, fornic and acetic acids. For the first group of dependant variables (P_{aq}, K_{aq}, P_{dw} and K_{dw}), the two iron types and the four fungal isolates were used. All observations in this group had a particle size E and the controls were excluded because of the very high values of P and K in the controls. This was as expected because of the complete MMN medium used in the controls, and the values obtained from the controls interfered with the proper

analysis and comparison of P and K in the different fungal treatments for this group of dependent variable.

The statistical analysis was designed to compare all levels of fixed factors for each dependant variable. The analyses carried out therefore included a 2-way ANOVA (4 fungal type X 2 iron type) for P_{aq}, K_{aq}, P_{dw} and K_{dw}, and a 3-way ANOVA(4 fungal type X 2 iron type) for K_{Loss} and P_{Loss}. For all other dependant variables, an ANOVA was conducted with a factorial 4X2X5 treatment structure to which 4 controls were added. The ANOVA table for this treatment structure was constructed by the way of contrasts to study the main effects of fungal type, Iron type and Particle size, their interactions and to test the difference between treatments and controls. Following a significant effect, multiple comparisons with the step down Bonferrori method were conducted to identify the differences between the treatments. This method was used to control for the type I error rate.

All the assumptions of any Anova model were verified. The homogeneity of variances was verified using the Bartlett's test and the normality assumption was tested with Shapiro-Wilk's statistic. For all the organic acid models, a logarithmic transformation was applied to the data in order to attain normality and homogeneity of variances.

3.3 Results

3.3.1 Mobilisation of K and P in relation to particle size and organic acid production

The XRF analyses of the two Sishen iron ores revealed their chemical composition to contain the following (average of six samples) - SiO_2 (31.6%), Al_2O_3 (2.8%) and Fe_2O_3 (61.30%) and trace values of TiO_2, CaO, MgO, Na_2O, MnO, Cr_2O_3, NiO, V_2O_5 and ZrO_2 for SK. Furthermore, KGT had SiO_2 (4.87%), Al_2O_3 (3.26%) and Fe_2O_3 (90.70%). Trace values of TiO_2, CaO, MgO, Na_2O, MnO, $Cr2O_3$, NiO, V_2O_5 and ZrO_2 were also present in KGT ore type. The SK ore type contains average values of 0.56% and 0.0685 % for K_2O and P_2O_5 respectively while KGT has average values of 0.995% and 0.152% for K_2O and P_2O_5 respectively. The major K_2O bearing mineral contained in both SK and KGT ores was characterised as muscovite by Sishen iron ore company (Richards, 1990 - 1992). The P-bearing minerals are apatite, woodhouseite, goyasite and gorceixite for KGT ore type, while those for SK has not been characterised because of low levels of P contained in this ore (Richards, 1990 -1992; Ogilvie, 2002).

However, full mineralogical characterisation of the Sishen iron ore is not available but from past data obtained through unpublished work, the following were listed: KGT contains minerals such as haematite, greenalite, muscovite, nacrite, apatite, woodhouseite, goyasite and gorceixite. For SK, minerals such as haematite, biotite, greenalite, illite, muscovite, nacrite, magnetite, quartz and siderite were listed. Fractions of these minerals contained in the mineral ores can differ from one site to another but haematite is the major mineral contained in both KGT and SK, while biotite is present as a minor mineral for SK ore type. All other minerals are in trace quantities and they may not be present in each of these minerals at all times.

The XRF results were calculated in terms of percentage quantity of K and P removed from the iron ore. The mobilisation was found to be significantly affected by fungal type,

ore type and particle as well as some of their interactions (Table 3.1) but could not be linked directly to the organic acid production (Fig. 3.1, Fig. 3.2 and Fig. 3.3). In both ore types, particle size was discovered to have "restricted effect" on the mobilisation of K and P. This effect was most observed in treatments involving particle size E where all the fungi used produced significant removal of K from the iron ore (Fig. 3.1A and 3.1B).

Table 3.1: Three-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type, mineral type, particle size and their interactions on %K and % P reduction from the iron ore materials.

Sources of Variation	**df**	**% K loss**		**%P loss**	
		df= 109		**df= 97**	
		F	*P*	*F*	*P*
Fungal type	3	32.63	<.0001	25.24	<.0001
Iron type	1	45.07	<.0001	475.85	<.0001
Fungal type vs Iron type	3	54.46	<.0001	37.87	<.0001
Particle size	4	209.11	<.0001	28.13	<.0001
Fungal type vs Particle size	12	8.72	<.0001	8.36	<.0001
Iron type vs Particle size	4	51.99	<.0001	3.27	0.0147
Fungal vs Iron type vs Particle size	12	11.63	<.0001	6.2	<.0001
Fungal type	3	32.63	<.0001	25.24	<.0001

P values <0.001 are considered significant.

Mobilisation of K from KGT treatment was best in particle size E (Fig. 3.1A) in all the ECM fungi with no significant difference among the fungal treatments. Values recorded here are far from the value needed to meet the commercial standard value of 75.88%. Meanwhile, for SK ore type, only PFR (Fig. 3.1B) was able to mobilise enough K (65.40%) from the iron ore, which is significantly above the commercial standard of 57.14%. There is however, no significant difference in the highest K leached from ST, PT and PI from particle size E treatment of SK ore type (Fig. 3.1B).

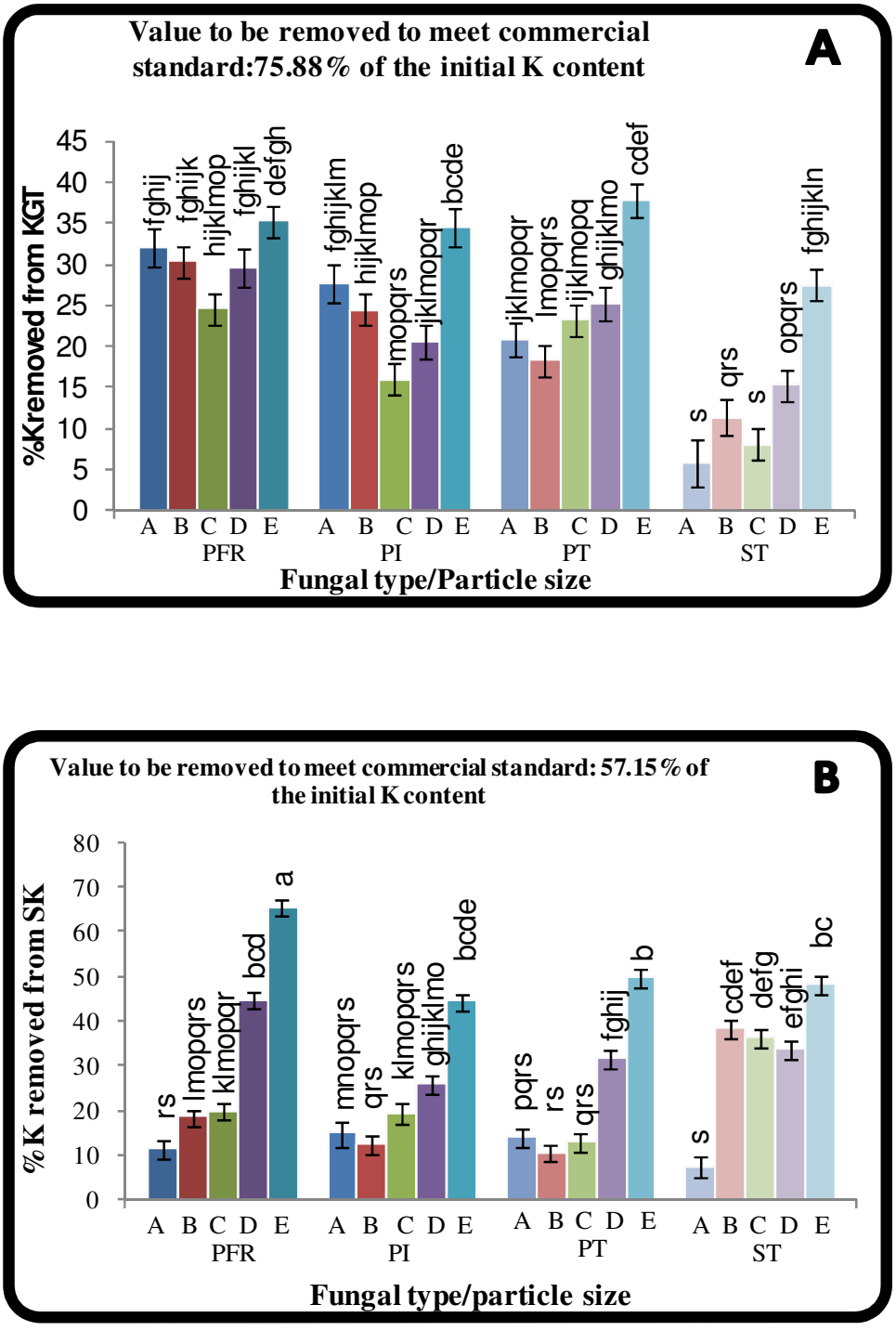
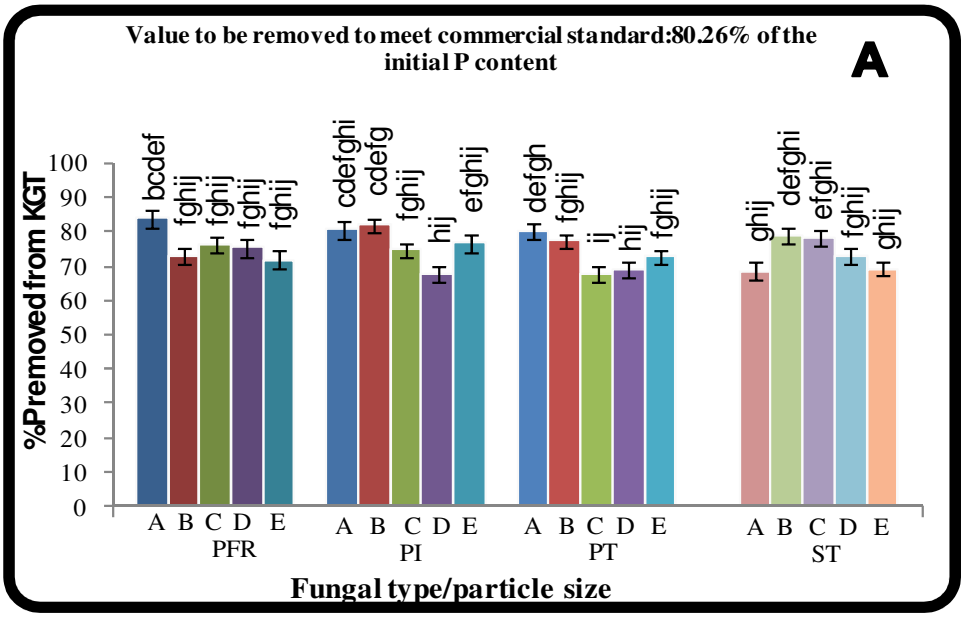


Figure 3.1: Percentage of K reduction from mineral type KGT (**A**) and mineral type SK (**B**) in response to the four different fungal treatments (PFR, PI, PT and ST) using five particle sizes (A,B,C,D and E) of the iron ore materials. Bars with the same letter are not significantly different. (P<0.0001).

The mobilisation of P seemed not to be affected much by particle size (Fig. 3.2 A and 3.2B) except in SK treatment where PI and ST produced less mobilisation from the smaller particle size C, D and E (Fig. 3.2 B). In general, better P removal in the iron ore was recorded in larger particle sizes of the iron ore (Fig. 3.2 A and 3.2 B). Only PFR, PI and PT (particle size A) were able to reduce the P level beyond the commercial standard (80.26%) in KGT treatment (Fig. 3.2 A). In addition, all the fungi reduced the P levels in SK beyond the commercial standard of 56.21% (Fig. 3.2 B). It is also important to mention that the least value of total Fe recorded in both ore types after the leaching experiment was >60%.



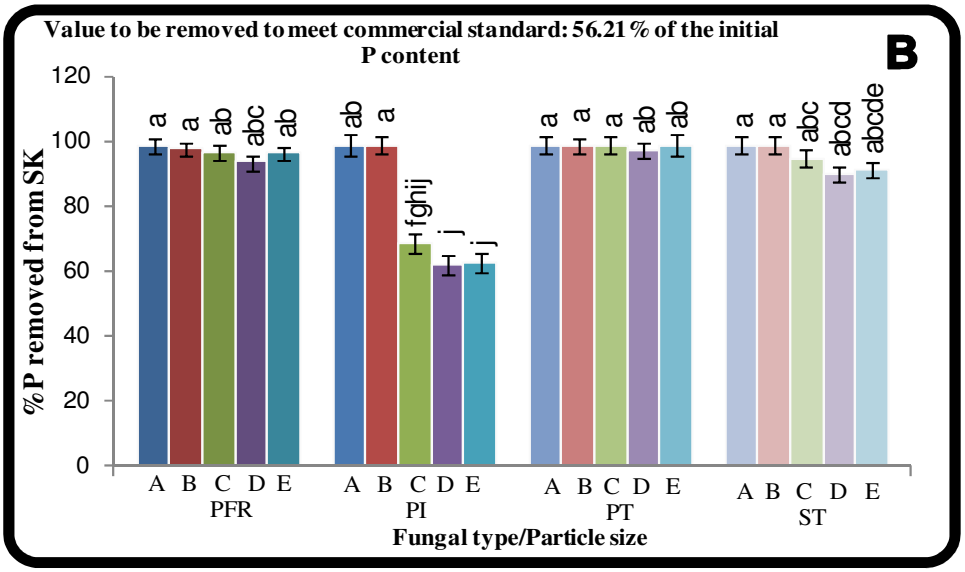


Figure 3.2: Percentage of P reduction from mineral type KGT (A) and mineral type SK (B) in response to the four different fungal treatments (PFR, PI, PT and ST) using five particle sizes (A,B,C,D and E) of the iron ore materials. Bars with the same letter are not significantly different. (P<0.0001).

3.3.2 Organic acid production

All the seven organic acids tested were detected in the medium of growth for all the four ECM fungi studied. As shown in Table 3.2, the release of organic acid was found to be significantly affected by the interactions among the factors: particle size, ore type and fungal type.

Table 3.2: One-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type, mineral type, particle size and their interactions on the release of seven different organic acids.

Sources of Variation		**Oxalic acid**		**Citric acid**		**Maleic acid**		**Malonic acid**		**Lactic acid**		**Formic acid**		**Acetic acid**	
		df= 124		**df= 125**		**df= 122**		**df= 122**		**df= 125**		**df= 128**		**df=116**	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fungal type	3	710.38	<.0001	655.73	<.0001	279.64	<.0001	570.95	<.0001	1034.5	<.0001	79.35	<.0001	2.83	<.0001
Iron ore type	1	0.96	0.3285	21.83	<.0001	55.87	<.0001	9.02	0.0032	5.45	.021	8.31	<.0001	07.5	<.0001
Particle size	4	273.46	<.0001	450.94	<.0001	171.77	<.0001	37.77	<.0001	190.9	<.0001	9.78	<.0001	64.79	<.0001
Fungal type vs Iron type	3	68.94	<.0001	35.18	<.0001	100.23	<.0001	86.59	<.0001	31.63	<.0001	101.33	<.0001	79.67	<.0001
Fungal type vs Particle size	2	33.63	<.0001	16.8	<.0001	26.06	<.0001	28.3	<.0001	80.33	<.0001	2.25	<.0001	37.06	<.0001
Iron type vs Particle size	4	13.08	<.0001	5.21	<.0001	5.19	0.0007	25.38	<.0001	43.02	<.0001	9.46	<.0001	12.01	<.0001
Fungal type vs Iron type vs Particle size	2	14.02	<.0001	46.23	<.0001	6.23	<.0001	21.6	<.0001	13.2	<.0001	21.68	<.0001	56.82	<.0001
Iron vs Control	4	70.18	<.0001	2.37	<.0001	32.94	<.0001	9.1	<.0001	123.53	<.0001	34.29	<.0001	5.73	<.0001

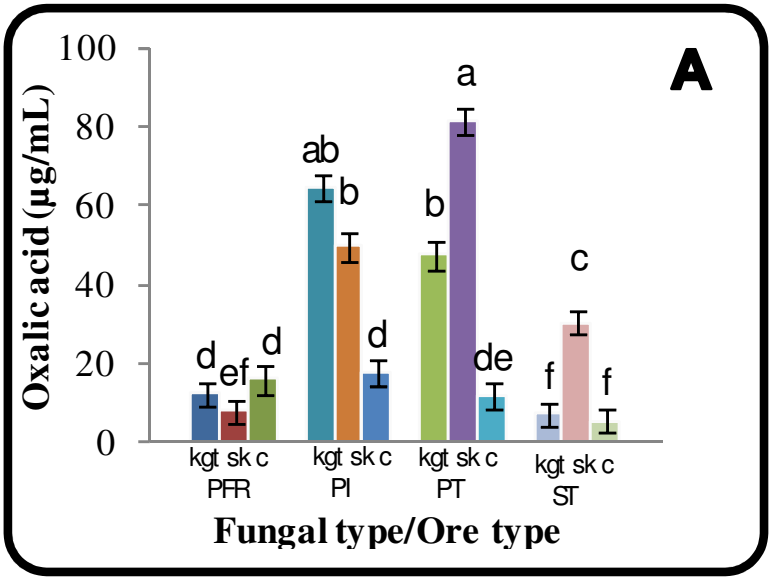
P values <0.0001 are considered significant.

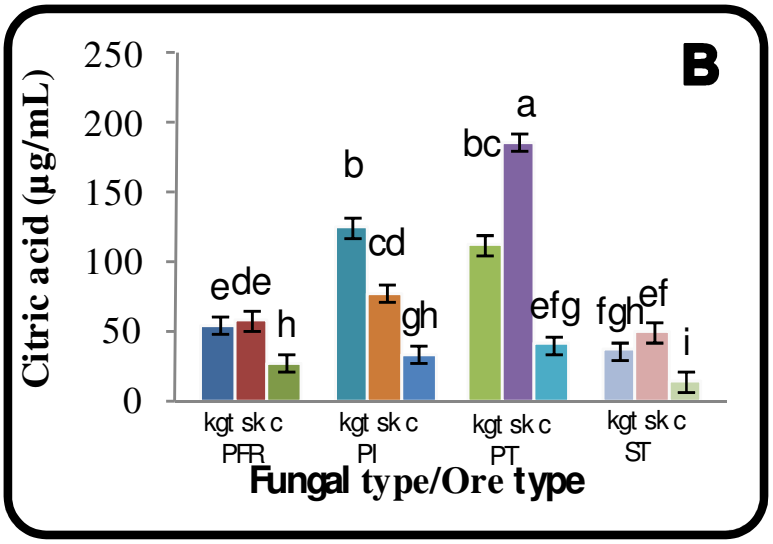
Table 3.3: Two-way analysis of variance (ANOVA) with F and P values that show the effects of the fixed factors – fungal type and mineral type as well as their interactions on the K and P accumulated in the spent broth and fungal dried mycelia.

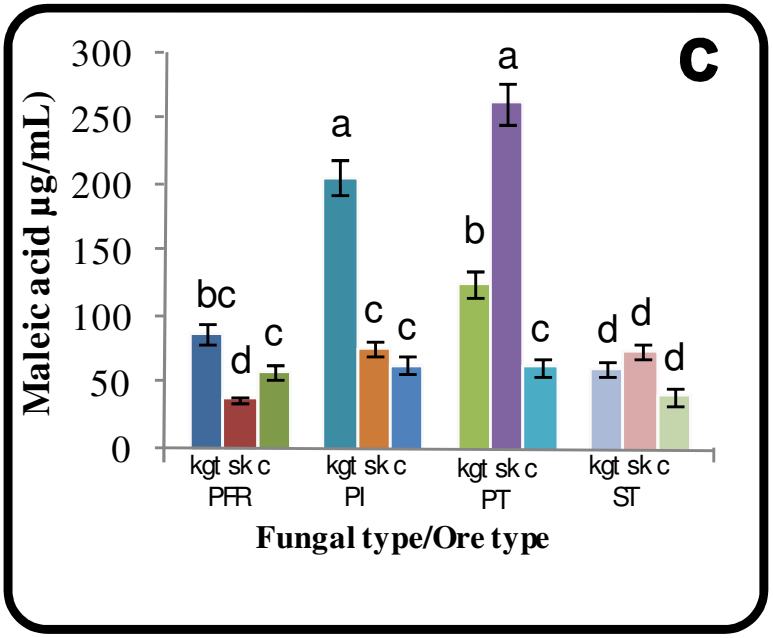
Sources of Variation	**df**	**Paq**		**Kaq**		**Pdw**		**Kdw**	
		df= 24		**df= 24**		**df= 24**		**df= 24**	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fungal type	3	49.46	<.0001	102.82	<.0001	32.31	<.0001	20.26	<.0001
Iron ore type	1	12.92	0.0015	3.07	0.0927	0.64	0.4326	26.13	<.0001
Fungal type vs Iron ore type	3	6.94	0.0016	7.1	0.0014	5.74	0.0042	17.84	<.0001

P values <0.0001 are considered significant.

In general, largest quantities of all the acids were recorded in the experiments involving the lowest particle sizes D, E and C of the two iron ore types (Fig. 3.3). The quantity was significantly higher in particle size D than E and C for oxalic, citric, malic, malonic and lactic acids, while the highest was recorded in particle size E in formic and acetic acids (Fig.3.3).






C
Maleic acid µg/mL)
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50
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PT
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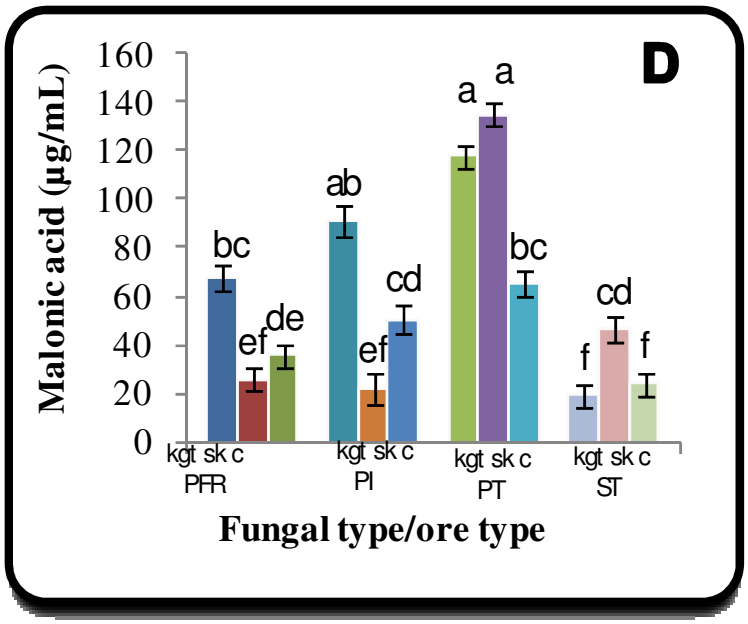
ST
Fungal type/Ore type


D
Malonic acid (µg/mL)
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120
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60
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20
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PT
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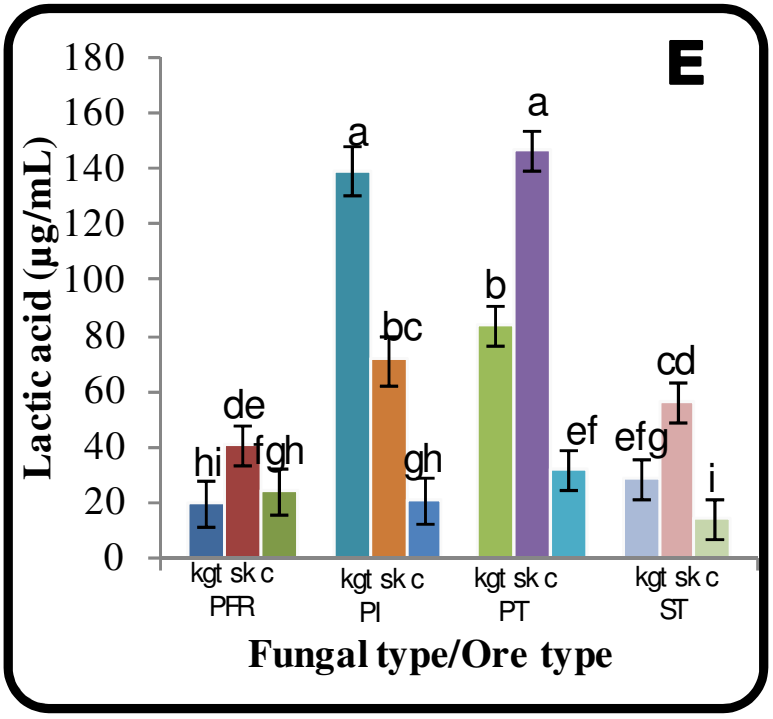
ST
Fungal type/ore type


E
Lactic acid (µg/mL)
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160
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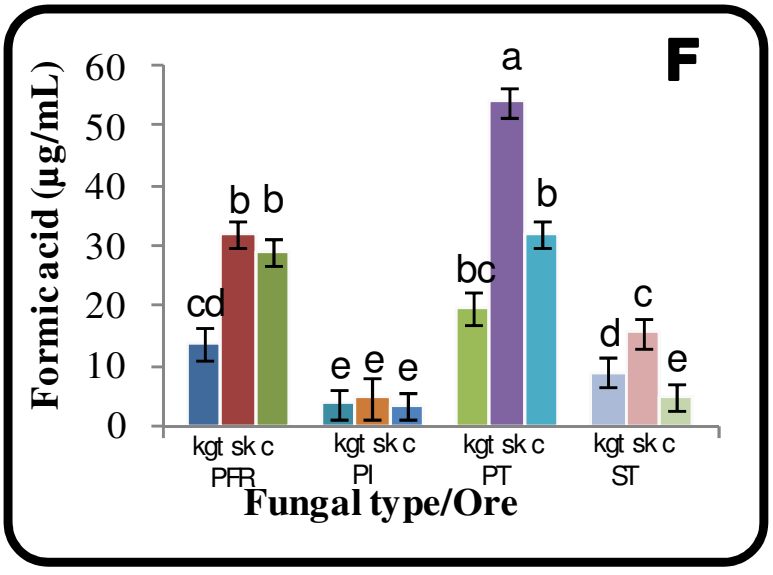
ST
Fungal type/Ore type


F
Formic acid (µg/mL)
60
50
40
30
20
10
0
cd
b b
e e e
bc
a
b
d
c
e
kgt sk c
PFR
kgt sk c
PI
kgt sk c
PT
kgt sk c
ST
Fungal type/Ore

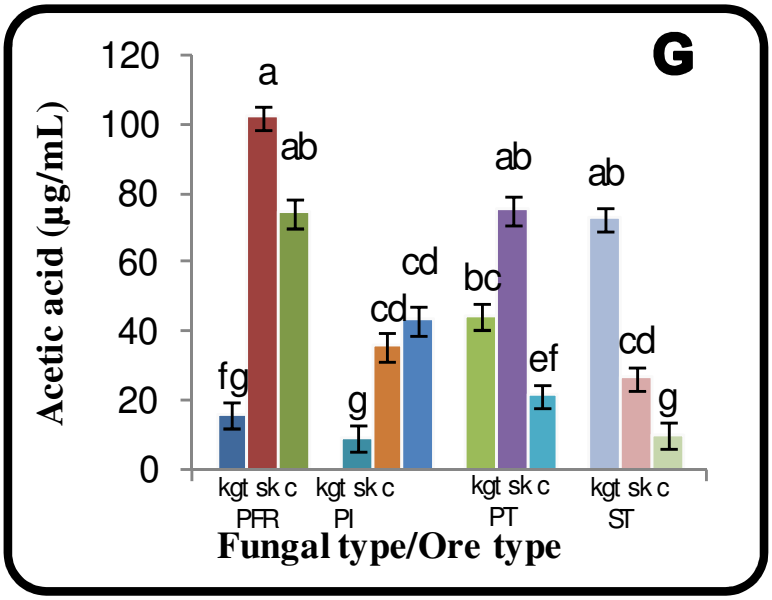


Figure 3.3: Amount of Oxalic acid (A), Citric acid (B), Maleic acid (C), Malonic acid (D), Lactic acid (E), Formic acid (F) and Acetic acid (G) released by the four ECM fungi (PFR, PI, PT and ST) grown under either KGT or SK treatments, or control – c (without fungus), with particle size E. Bars with the same number are not significantlydifferent ($P<0.0001$).

By comparing the organic acid released in particle size E treatment where the highest mobilisation of K occurred, it was revealed that PT released the highest quantity of oxalic acids in both iron types. The same trend was repeated in all other types of organic acids studied, except citric acid where the highest quantity was produced by PFR in SK treatment (Fig. 3.3). Contrary to expectation, the mobilisation of the two types of the iron ore does not correspond to organic acid detected in their respective medium of growth. The highest K reduction in particle size E treatment was from PFR, but this does not correlate with the quantity of organic acid released by the same fungus (Fig. 3.3).

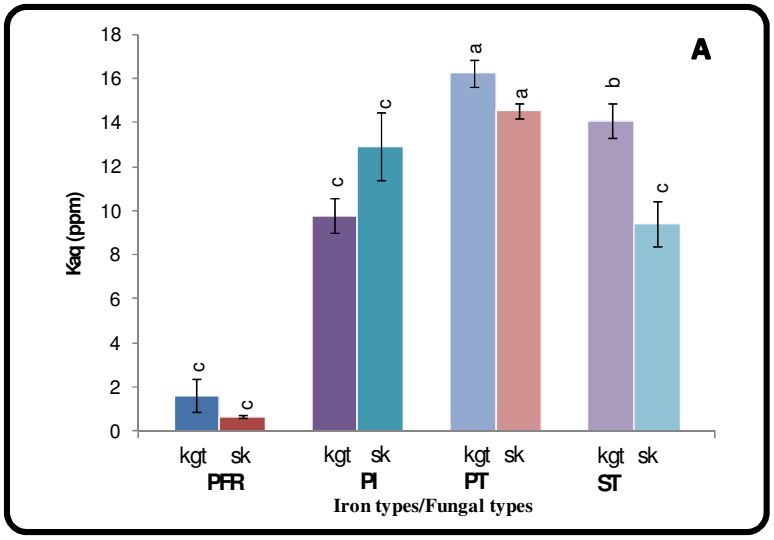
High quantity of acetic acid produced by PFR in particle size E corresponds to the highest leaching percentage of this fungus (Fig. 3.1, Fig. 3.2 and Fig. 3.3G). However, a claim that this acid could be solely responsible for the mobilisation of K could not be verified because of lack of similar evidence in the other ECM fungi that produced high

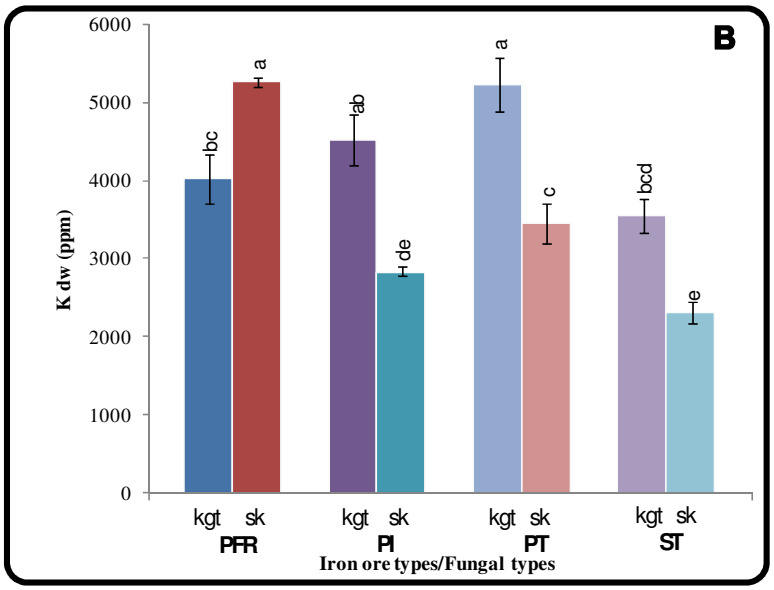
acetic acid Fig. 3.1, Fig. 3.2 and Fig. 3.3G). For example, high acetic acid production by PT and ST (Fig. 3G) did not translate into better mobilisation of K as shown in Fig. 3.1A and 3.1B.

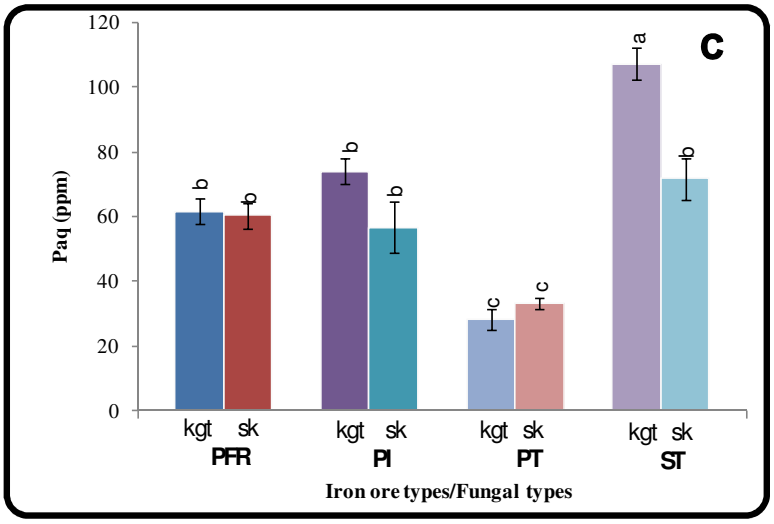
3.3.3 Aqueous K /P and Dry mycelia K/P

The inability to properly link production of organic acid to the mobilisation of K and P from the two iron ore types led to further investigation where K and P contents of both the growth medium and fungal mycelia were analysed. The analysis was carried out only for treatment with particle size E where the best leaching was produced. There were significant effects of fungal type on the amount of K and P detected in aqueous and dried mycelia culture of all the four fungi for treatments involving the two ore types (Table 3.3).

In iron ore type 2 (SK), high quantity of aqueous K was discovered in PT growth medium while the lowest was discovered in PFR growth medium (Fig. 3.4 A).




B
K dw (ppm)
6000
5000
4000
3000
2000
1000
0
bc
a
ab
de
a
c
bcd
e
kgt sk
PFR
kgt sk
PI
kgt sk
PT
kgt sk
ST
Iron ore types/Fungal types


C
Paq (ppm)
120
100
80
60
40
20
0
b
b
b
b
c
c
a
b
kgt sk
PFR
kgt sk
PI
kgt sk
PT
kgt sk
ST
Iron ore types/Fungal types

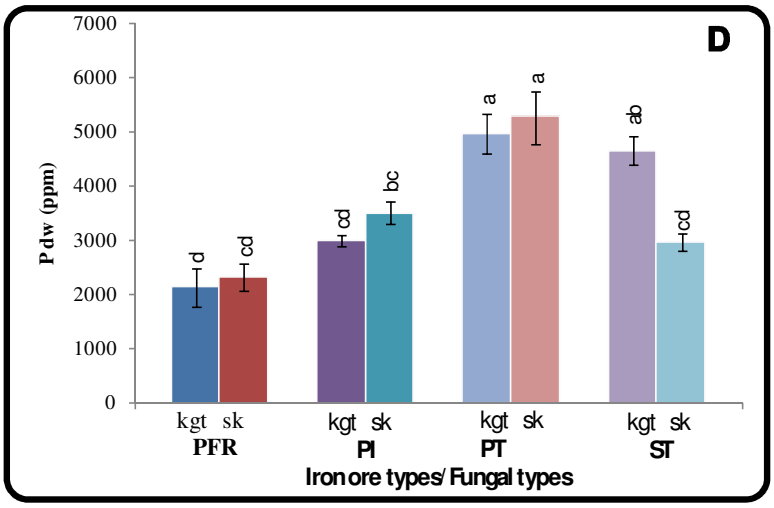


Figure 3.4: Amount of K measured in the growth medium (A) and in the dried mycelium (B) of each of the four fungi with either KGT or SK mineral type. Amount of K measured in the growth medium (C) and in the dried mycelium (D) of each of the four fungi (PFR, PI, PT and ST) with either KGT or SK mineral type. Bars with the same number are not significantly different (P<0.0001).

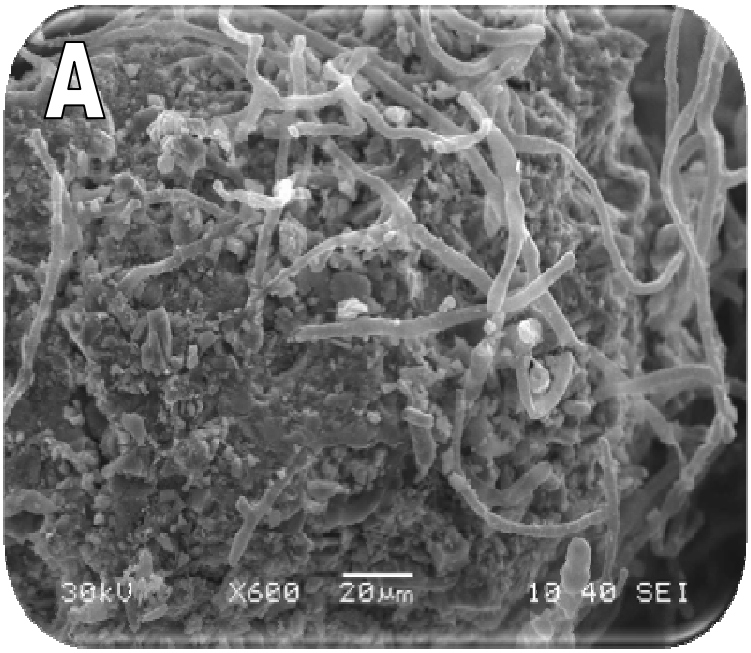
Contrary to this, dried mycelia culture of PFR had the highest concentration of K, whereas the K content of PT mycelia was lower (not significantly different) (Fig. 3.4 B). In addition, PT had the lowest aqueous P and highest mycelial concentration of P in both SK and KGT (Fig. 3.4 C and D) ore types, a reflection of the quantity of P that was mobilised from both ore types by PT. As expected, the controls had high K and P (aqueous and mycelial) contents and are not reported.

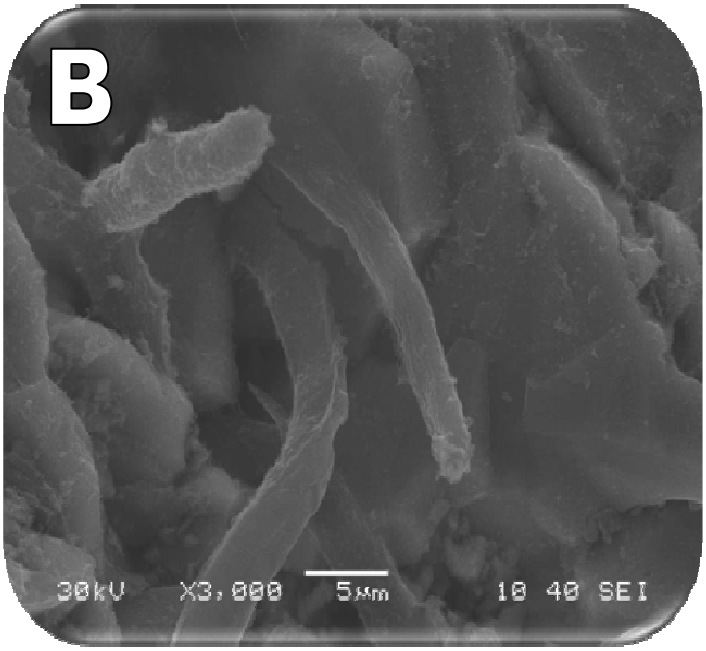
3.3.4 Microscopy

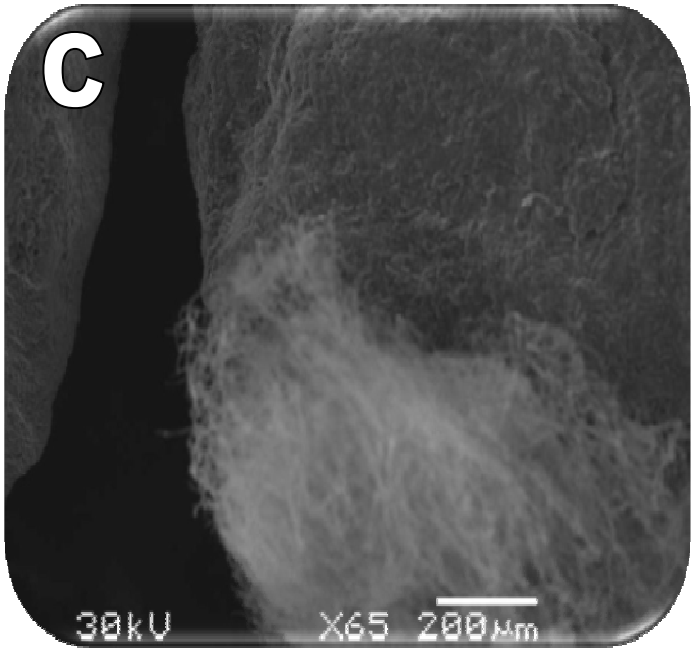
Physical contacts between iron ore and fungi were observed for all types of fungi, though at various levels. There was not much difference between the attachments of the fungi to the two ore types but there were differences among the fungi. The strongest attachment to the iron ore surface was recorded in PFR where mycelia of the fungus seemed to have the

capability of producing sticky materials that helped in proper binding of the mycelial to the iron ore (Fig. 3.5 A and 3.5B). Furthermore, these PFR mycelial structures looked as if they penetrated the ore (Fig. 3.5 B) but this could not be confirmed. In addition, preferential attachment of PT to phosphate rich mineral (Eudral goyasite crystals – as identified by Richards (1990 -1992) was observed on SK ore type (Fig. 3.5 C).

Attachment of ST was only to the surface of the iron ore and never showed any penetration of the ores by the mycelial structures (Fig. 3.5 D). Meanwhile, PI was observed to attach moderately to the iron ore materials (Fig. 3.5 E).




B
30kV X3,000 5µm 10 40 SEI


C
30kV X65 200µm

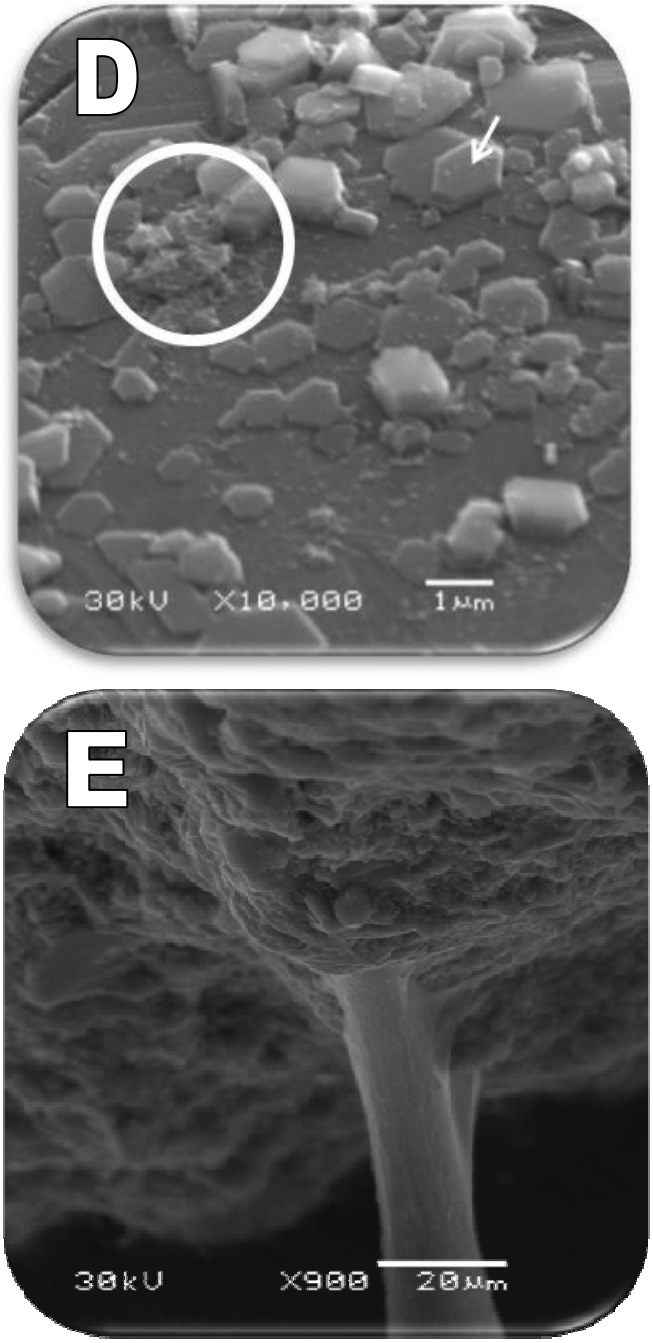


Figure 3.5: A and B represent the SEM images of PT mycelia with scattered attachments to the surface of the iron ore, no sign of penetration of the ore was visible. C and D represent the SEM images of PFR with strong attachment to the iron ore and possibility of penetrating the iron ore (D). E represents the SEM images showing slight attachment of ST mycelia to the iron ore surface. F represents the PI attachment to the surface of the iron ore.

3.4 Discussion

Studies have been able to show the ability of ECM fungi to mobilise nutrients from different minerals under different conditions (Paris *et al*., 1996; Yuan *et al*., 2004; van Scholl *et al*., 2006b). Most of these studies involved the use of mycorrhizal plants as well as single type of K and P bearing minerals such as feldspar, biotite, muscovite and apatite (Wallander and Wickman, 1999; Yuan *et al*., 2004; van Scholl *et al*., 2006b; Balogh-Brunstad *et al*., 2008; Calvaruso *et al*., 2009). However, this study has investigated the mobilisation of nutrients from mineral ore that consists of one or more of such minerals using ECM fungi.

Omission of some key nutrients in the medium of growth has probably helped in the leaching process by triggering the production of organic acids and creating a need for the fungi to source for the missing nutrients from the ores. Similar methods that involved the use of nutrients poor solution in the presence of insoluble minerals have also been adopted in previous related studies (Paris *et al*., 1996; Leake *et al*., 2008; Calvaruso *et al*., 2009). Although biomass was not taken into consideration in this study, visual observation suggested higher biomass of ECM fungal mycelia in the presence of iron ore samples compared to the controls with no addition of iron ore. Such observation has been initially reported by Wallander and Thelin (2008) that addition of minerals such as apatite stimulates the growth of ECM fungi.

Particle size of the ore, production of organic acids by the fungi, and ore types were all observed to significantly affect the nutrient mobilisation. Reduction of particle size increases the total particle surface area of minerals that is exposed to the leaching process and therefore is expected to increase the solubilisation rate (Bosecker, 1997; Vasan *et al*., 2001; Jain and Sharma, 2004). This fact was partially confirmed in this study with leaching of K from both ore types. Very fine particle size of the iron ore (<0.1 mm) led to more leaching than the coarser particle sizes. This was attributed to the ease of releasing

nutrients from such particle size, because of larger particle surface exposed. As discussed by Modak *et al*. (2001) and Jain and Sharma (2004), the use of small particle size minerals could save time and resources during commercial bioleaching processes.

However, data on particle size should be interpreted with caution as it is difficult to identify a general trend through which this factor affects mobilisation of nutrients from minerals. Although time effect was not investigated in this study, it is plausible that particle size is only relevant when time required for leaching is considered. In the study conducted by Modak *et al*. (2001), leaching of calcium from minerals with coarser particle size was similar to those from finer particle size but differ on the basis of time required for the leaching process. Results obtained from this study only indicate consistent "particle size E effect" on K mobilisation and not for other particle sizes investigated. This is in agreement with the suggestion of Modak *et al*. (2001) that recommended >0.1 mm size as optimum for bioleaching process. In spite of this fact, the deviation of P solubilisation from such a trend during this study, suggests that finer particle size may not be applicable to all mineral/ore leaching processes. Delvasto *et al*. (2009) reported a similar scenario where more P was leached from coarser ore particle (2 mm) as compared to those with smaller particle size (0.2 mm).

Fungal leaching largely occurs through four main mechanisms namely acidolysis, complexolysis, redoxolysis/reduction and metal accumulation (Burgstaller and Schinner, 1993). Most of these mechanisms are directly and indirectly related to the ability of fungi to produce organic acids and ligands (Burgstaller and Schinner, 1993; Jain and Sharma, 2004). For example, acidolysis results from a decrease in pH that can be caused by fungal exudation of organic acids as mentioned in a study conducted by Badr *et al*. (2006). In their investigation, they suggested that production of organic acids by silicate-solubilising bacteria lowered the pH to 3.5, which consequently improved the dissolution rate of K- and P-bearing minerals. In another study, reduction of Fe^{3+} to Fe^{2+} was credited to fungal production of oxalate by Ghiorse (1988).

Therefore, on the basis of such theories, the influence of organic acids on nutrient mobilisation was well acknowledged and investigated in this study. The only acid that was produced in higher quantity by PFR (removed highest quantity of K) was acetic acid. However, this tendency could not be linked to other isolates. Numerous studies (Paris *et al*., 1995; Lapeyrie *et al*., 1987; Parks, 1990; Wallander and Wickman, 1999; Rosling, 2004; Williams, 2008) had successfully linked biological weathering and leaching of minerals to the effects of organic acids. These acids act in two major ways that involve the contribution of metal complexing organic acid anion and protons (Gadd, 1999). High chelation constants of $[Al\ (C_2O_4)_3]^{3-}$; 2.0×10^{16} and $[Fe\ (C_2O_4)_3]^{3-}$; 3.9×10^{16} make mineral sites containing Al^{3+} and Fe^{3+} easy target for attack by organic acid anion. Oxalate chelation of these cations results in structural imbalance and therefore may lead to the dissolution and release of other elements embedded in minerals such as K and P (Yuan *et al*., 2004; Delvasto *et al*., 2009). Secondly, both protons (from organic acids) and K (from minerals such as muscovite) are monovalent, but the size of the protons (0.32×10^{-10} m) is much smaller than that of K (2.03×10^{-10} m) (Lapeyrie *et al*., 1987; Yuan *et al*., 2004). The size therefore gives an advantage to protons to replace interlayer K contained in layer minerals such as muscovite associated with iron ores investigated in this study.

Although this study did not specifically investigate solubilisation of any particular mineral type, as earlier mentioned, iron ore samples investigated contained minerals such as muscovite, biotite, goyasite and apatite. Mobilisation of nutrients from these minerals has been previously linked to effects of organic acid produced by microoganisms (Badr *et al*., 2006; Delvasto, 2009; van Scholl *et al*., 2006a; Sheng *et al*., 2008; Williams, 2008). Sheng *et al*. (2008) suggested that gluconic and acetic acid production by *Bacillus globisporus* influenced the release of K from feldspar mineral. Similar idea was pointed out by Delvasto *et al*. (2009) having utilised *Burkholderia caribensis* FeGL03 for beneficiation of iron ore materials. In their study, gluconic acid was noted to play a central role during the leaching process. Williams (2008) went a step further by utilising

Aspergillus niger to produce citric acid for leaching of iron ore materials. About 17.65% K and no P were removed by this process. Organic acids detected in the medium of growth in this study have therefore provided an important relationship to the aforementioned studies. However, results of statistical analyses obtained indicated that organic acids may not be the only reason for the dissolution of iron ore samples. These results could be an indication that though organic acid is important, it may not be the only reason for mineral solubilisation by ECM fungi.

The physical presence, as well as attachment of the fungus to mineral could be equally important for leaching process. In the initial physical observation of the growth inside the conical flask and during the microscopic observation, PFR was observed to strongly attach to both ore types. Despite the fact that penetration of the ore types by PFR could not be confirmed, such attachments are very important and are enhanced by slime production by ECM fungi (Denny and Wilkins, 1987; Gadd, 2000). This is one of the mechanisms that fungi generally used to solubilise minerals (Burgstaller and Schinner, 1993). Apart from the direct binding of elements from the minerals, attachment to mineral surfaces also enhance a particular feeding mechanism in fungi known as scavenging (Banfield *et al*., 1999; Delvasto, 2009). Closeness of the fungal mycelia to the mineral surface during scavenging promotes direct absorption of nutrients contained inside the mineral. In addition, the propinquity also allows faster action of organic acids on the minerals. Any or all these factors may contribute to increased solubilisation of iron ore through scavenging by the ECM fungi (Smith and Read, 2008).

Slime produced by microorganisms largely consists of polysaccharides that can bind metals from solution (Banfield *et al*., 1992). Such a process can possibly create a concentration gradient that can increase movement of ions from mineral into solutions (Delvasto *et al*., 2009). Likely evidence of this was confirmed by the K and P analyses of the aqueous growth medium and the dried mycelium of these fungi. For instance, high levels of K detected in the dried mycelium of PFR may indicate PFR was very effective

in utilising K released into the medium. This may be the reason for the low concentration of K in the growth medium and the high concentration of K in the mycelium. In the presence of organic acid exudation, such process can create a concentration gradient that could allow more K to be released into the solution in response to the low levels of K in solution. In addition, this may further explain the reason behind the lower K reduction for PT fungal type in SK ore type despite high organic acid production. Slow utilisation of K in the medium by PT must have slowed down the process of K release from the medium because of lack of "sucking pressure" that could create a concentration gradient for more K to be released.

Finally, attachments of fungi to specific sites on mineral surfaces, e.g. PT attaching to goyasite, is an indication that complete mineralogical characterisation of both KGT and SK iron ore types will provide more information for development of a reliable biobeneficiation method for these ores. In addition, the higher levels of both K and P contained in KGT iron type must have made it difficult for the fungi to reduce the concentration of both elements from this ore type. Repeated leaching or longer exposure could eventually reduce these elements beyond the commercial standards. It is important to mention that results obtained from this study could differ when the fungi are in symbiosis. However, the study has laid a foundation for future consideration of ECM fungi in biohydrometallurgical processes.

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