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Kalaharituber pfeilii and associated bacterial interactions

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ABSTRACT

Truffles are generally known to form a mycorrhizal relationship with plants. *Kalaharituber pfeilii* (Hennings) Trappe & Kagan-Zur is a species of desert truffle that is found in the southern part of Africa. The life cycle of this truffle has not been fully investigated as there are many unconfirmed plant species that have been suggested as potential hosts. Many mycorrhizal associations often involve other role players such as associated bacteria that may influence the establishment of the mycorrhizal formation and function. As part of an effort to understand the life cycle of *K. pfeilii*, laboratory experiments were conducted to investigate the role of ascocarp associated bacteria. Bacterial isolates obtained from the truffle ascocarps were subjected to microbiological and biochemical tests to determine their potentials as mycorrhizal helper bacteria. Tests conducted included stimulation of mycelial growth in vitro, indole acetic acid (IAA) production and phosphate solubilising. A total of 17 bacterial strains belonging to the Proteobacteria, Firmicutes and Actinobacteria were isolated from the truffle ascocarps and identified with sequence homology and phylogenetic methods. Three of these isolates showed potential to be helper bacteria in at least one of the media tested through the stimulation of mycelial growth. Furthermore, four isolates produced IAA and one was able to solubilise $CaHPO_3$ in vitro. One isolate, identified as a relative of *Paenibacillus* sp. stimulated mycelial growth on all the media tested. Other bacterial isolates that showed potential stimulation of mycelial growth were identified molecularly as a *Bacillus* sp. and two strains of *Rhizobium* sp. This study has contributed to the existing knowledge on the biotic interactions with *K. pfeilii* which may be useful in further symbiont and re-synthesis investigations.

1. Introduction

Kalaharituber pfeilii (Hennings) Trappe & Kagan-Zur (formerly *Terfezia pfeilii*) (Ferdman et al., 2005), is an edible mycorrhizal fungus that thrives in the Kalahari desert of Southern Africa (Taylor et al., 1995), and is commonly referred to as the Kalahari truffle or n'abba. This fungus is a desert truffle that belongs to the family Terfeziaceae (Percudani et al., 1999). *K. pfeilii* cannot complete its life cycle without its host plants (Taylor and Alexander, 2005). Identification of the hosts is challenging as little information is available regarding the general biology of this fungus (Kagan-Zur et al., 1999). However, other factors which may directly assist in the mycorrhizal establishment of *K. pfeilii* can be investigated.

It is increasingly apparent that mycorrhizal associations are not only between plant and fungus but includes other microorganisms that inhabit the rhizosphere (Garbaye, 1994). There have been several investigations highlighting the positive, negative and neutral effects of soil microorganisms, especially rhizobacteria, on mycorrhizal formation (Poole et al., 2001; Sbrana et al., 2002). This has led to a proposed tripartite association between the plant, mycorrhizal fungus and those bacteria that exhibit positive effects on the association (Poole et al., 2001). These bacteria are referred to as Mycorrhization helper bacteria (MHB) (Garbaye, 1994). Survival and existence of these bacteria in the rhizosphere have been attributed to the presence of plant root exudates that contain essential nutrients for their growth (Smith and Read, 2008; Suresh and Bagyaraj, 2002).

The mode of action of MHB is not well understood but Garbaye (1994) proposed various bacterial mechanisms which may aid mycorrhizal formation. These included the effect of MHB in stimulating fungal growth and the production of substances by the MHB that facilitates fungal penetration of the host root and/or recognition of the root by the fungus. In addition, the production of metabolites such as growth hormones that increase the short root formation in plants, thereby increasing the chances of fungal contact and improving establishment of the mycorrhizal association was also suggested.

Research on the biology of *K. pfeilii* has been focused on determining possible host plants with little attention being paid to potential role players such as MHB. The present study investigated the culturable bacteria associated with the ascocarps of *K. pfeilii*. Based on the performances of these bacterial isolates during investigation, some isolates were selected and identified using molecular methods. Beneficial effects, if present, could indicate the potential of these bacteria to be MHB. Various objectives set to achieve this aim were investigated by first isolating the bacteria from the ascocarp of *K. pfeilii*, followed by subsequent investigations into MHB characteristics of these bacteria such as

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IAA production, in vitro assay of bacterial activity on mycelial growth and phosphate solubilising capability.

2. Material and methods

2.1. Bacterial Isolation

K. pfeilii hypogenous fruiting bodies (ascocarps) were collected from the Spitskop Nature Reserve, Upington, Northern Cape province, South Africa (S28° 22.780′, E21° 09.420′). Surface-sterilised ascocarps were split open and internal explants of between 10 and 15 mg were removed and inoculated onto a Fontana (FTN) medium (Fontana, 1968). Bacterial colonies originating from explant material were subcultured onto Nutrient agar, coded and are maintained as glycerol stocks and stored at −80 °C in the Mycorrhizal Research Laboratory collection. Gram staining was performed on all the isolates as part of the identification process and to ensure purity (Madigan et al., 1998).

Because the host plants of *K. pfeilii* had not been positively identified the use of mycorrhizal re-synthesis experiments could not be exploited for this study. Therefore in order to establish whether the associated bacteria had any potential to assist in mycorrhization various in vitro assays were adopted.

2.2. Co-culture of bacterial isolates and K. pfeilii

Three different media were used: Fontana medium (FTN), 1.5% water agar (WA) and poor Fontana medium (PFTN) with reduced concentrations of glucose from 6.5 to 2.25 g L^{-1} (Molina and Palmer, 1982). Bacterial samples were inoculated into nutrient broth (NB) and grown at 37 °C overnight. The cultures were centrifuged at 13,000 rpm after which the supernatant was discarded. The cells were re-suspended in sterile double-distilled water. The concentrations of all the bacterial isolates were adjusted (Sbrana et al., 2002) with sterile water using a Beckman spectrophotometer (Du® 530) at wavelength of 600λ to an OD range of between 0.300 and 0.400 (Poole et al., 2001).

Four perpendicular wells equidistant from the centre of the Petri dish were created on each plate with a 5 mm corer. A bacterial concentration volume of 20 μL was placed into each well, giving a total volume of 80 μL of each bacterial sample per plate. A plug of *K. pfeilii* culture grown on an FTN medium was removed and inoculated at the centre of each plate. Three replicates were prepared for each bacterial sample tested. The plates were incubated in the dark at 32 °C, which is the optimal temperature for growth of *K. pfeilii* (Adeleke, 2007). The fungal colony diameter was recorded daily along the two perpendicular axes over a period of 14 days. The bacterial effects were evaluated by comparison with a control which contained sterile water in the wells.

There were two additional treatments that included only PFTN and WA. The low level/absence of essential nutrients in these media allowed the stimulatory effects of the bacterial isolates to be effectively monitored (Garbaye, 1994). The bacterial concentration had to be adjusted to a reduced OD_{600} reading of 0.03 to 0.04 because of the rapid growth of some isolates.

Further investigations were conducted on a selected isolate to establish the effect of different concentrations of the bacterial isolate on fungal growth using FTN, PFTN and WA. Two different concentrations of this isolate were used by adjusting the OD_{600} reading with sterile water to 0.05 and 0.50.

2.3. Indole acetic acid production and phosphate solubilisation

The ability of all the bacterial isolates to produce indole acetic acid (IAA) was investigated using the method of Gordon and Weber (1951). A liquid medium containing a 1:1 ratio of NB and tryptone powder was used for the culturing of the bacterial isolates. Tryptone powder is a source of L-tryptophan which is a precursor for the formation of IAA (Ahmad et al., 2005). Bacterial isolates were inoculated into the broth and incubated for 96 h in the dark at 37 °C. The cultures were centrifuged at 4500 rpm for 20 mins. The supernatant (300 μL) was tested for the presence of IAA using 600 μL of a Salkowski reagent (Gordon and Weber, 1951). A colour change of the reagent from yellow to red in the mixture was positive indication of IAA production.

A selective medium for phosphate solubilising microoganisms (Mehta and Nautiyal, 2001) was modified by replacing $Ca_3(PO_4)_2$ with $CaHPO_3$ clearing zones around bacterial colonies indicated positive solubilising potential.

2.4. Molecular identification and phylogenetic analysis

Bacterial isolates were selected for molecular identification based on the stimulatory or inhibitory activities observed in dual culture. Genomic DNA was extracted from overnight NB cultures of the bacterial isolates using the phenol/chloroform extraction method as described by Moore et al. (1987). The 16S rDNA bacterial genes was the target region for the PCR amplification using a pair of bacterial forward and reverse primers; GM5F (5 -CCTACGGGAGGCAGCAG-3′) and R907 (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTT-GAGTTT-3′), respectively (Moore et al., 1987; Myers et al., 1985). PCR was conducted in a 50 μL volume which contained the following: 0.4 μM of each of the primers, 1.25 units of *Taq* polymerase, 5 μL of a Promega 10× buffer (0.2 mM) Promega dNTPs, 1.75 mM of magnesium chloride and 2 μL template DNA. The PCR was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad). PCR products were separated electrophoretically with ethidium bromide (0.1 μg/mL) stained 1% agarose gel. The PCR products were allowed to run at 120 V for 60 mins. DNA was visualised and photographed using an UVIprochem Transilluminator.

The PCR product obtained was cleaned using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) and resuspended in 30 μL of nuclease-free double distilled water. The cleaned PCR product was sent to the Rhodes University Sequencing Facility in South Africa. The product was sequenced using an ABI prism BigDye Terminator v3.1 Ready Reaction Cycle sequencing kit (Cat.# 4336917) according to the manufacturer's instructions. The sequences were electrophoresed on an AB3100 Genetic Analyser (Applied Biosystematics). The forward and reverse DNA sequences obtained were aligned using BioEdit software (Hall, 1999). The sequences were BLASTed on the NCBI website (Wheeler et al., 2006) to confirm the nearest identical organism. Genera obtained from the GenBank with high percentage identity (>95%) were assumed to be very significant while values >98% were chosen to be significant at the species level in this study.

Relevant identical nucleotide sequences (two for each isolate) were obtained from the GenBank. Thereafter, nucleotide sequences of all the isolated bacteria strains and their identical relatives obtained from the GenBank were subjected to alignment using Mafft software (Katoh et al., 2002). The aligned sequences were used for the phylogenetic analysis using Mega 4 software and the evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2007). *Cupriavidus necator* was used as an out group and a neighbour joining (NJ) method was performed to infer the evolutionary history of the isolates. The bootstrap consensus tree was inferred from 1000 replicates.

2.5. Statistical analysis

Data obtained from in vitro assays of the bacterial activity on mycelial growth of *K. pfeilii* were analysed using a repeated measure ANOVA. This type of ANOVA is used when samples of the same characteristics are measured under different conditions. In this case, the fungal colony diameter is the measurement while day is the condition that changed. Fischer LSD was then selected to evaluate the level of significance. Statistica 7 software was used for the analysis (StatSoft, 2005).

3. Results

3.1. Bacterial isolation

Seventeen different bacterial isolates were obtained and coded. From the Gram stain, a total of 10 Gram positive and 7 Gram negative isolates were identified. Bacterial cells observed under the microscope revealed that 7 of the Gram positive bacteria were rods while 3 were cocci. For the Gram negative, 4 were rods, 2 were cocci and 1 bacillus (Table 1).

3.2. Co-culture of bacterial isolates and K. pfeilii

Plate readings were recorded from the 4th to the 14th day of the experiment. The bacterial isolates exhibited various effects ranging from neutral, inhibitory to stimulatory on the growth of the mycelial culture of *K. pfeilii*. As presented in Table 2, isolate KB06F1 had the highest significant stimulating effects on the growth of the fungus on the FTN medium (Fig. 1). A similar result was recorded for isolate KB06K with a significant stimulatory effect on growth of the mycelial culture although the effect was slow initially (Table 2).

Isolates KB06A, KB06C, KB06G, KB06H, KB06I and KB06J had neutral effects on the growth while KB06D, KB06E, KB06F2, KB06L, KB06NA, KB06NC, KB06ND, KB06NE and KB06NF inhibited the proliferation of the mycelial growth (Table 2). KB06NC and KB06ND initially produced stimulatory effects on the growth of the fungus until the seventh and eighth day, when their effects gradually became inhibitory (Table 2).

On the PFTN medium, 6 of the isolates; (KB06D, KB06F2, KB06G, KB06L, KB06NE and KB06NF), produced significant inhibitory effects while 11 isolates; (KB06A, KB06C, KB06E, KB06F1, KB06H, KB06I, KB06J, KB06K, KB06NA, KB06NC and KB06ND), produced neutral effects on the growth of the mycelia culture (Table 2). On WA, 13 out of 17 isolates produced neutral effects on the growth of the fungus while KB06NC was the only isolate that exerted a significant stimulatory effect on the growth of the fungus (Table 2). Four isolates, (KB06A, KB06G, KB06ND and KB06NF) were inhibitory and KB06NF did not allow any growth of the fungus (Table 2). Isolate KB06NF remained inhibitory to the growth of the fungus on all the media used. The stimulatory effect of the isolate KB06F1 was further investigated by conducting a concentration dependent experiment on WA and PFTN media. The result revealed that the stimulatory effect of this isolate at a higher concentration ($OD_{600} = 0.5$) was significantly greater than the effect at a lower concentration (Table 3).

Table 1
Characteristics of bacterial isolates associated with *Kalaharituber pfeilii* and sequence identity of the 16S rRNA sequence of selected bacterial isolates.

Bacterial isolate	Gram reaction/ shape	Indole test	P solubilisation	GenBank accession number	Most significant alignment	% Identity
KB06A	−cocci	+	−			
KB06C	+cocci	−	−			
KB06D	−cocci	+	−			
KB06E	+cocci	−	−			
KB06F1	+rods	−	−	EF589815 (29)	*Paenibacillus lautus*	96%
KB06F2	+rods	−	−			
KB06G	−rods	+	−	EF589810 (34)	*Rhizobium tropici*	98%
KB06H	+rods	−	−			
KB06I	+cocci	−	−			
KB06J	−rods	−	−			
KB06K	+rods	−	−	EF589814 (49)	*Bacillus* sp.	98%
KB06L	+rods	−	−	EF589812 (49)	*Curtobacterium* sp.	99%
KB06NA	+rods	−	−			
KB06NC	−rods	+	−	EF589811 (34)	*Rhizobium tropici*	99%
KB06ND	−rods	−	−			
KB06NE	+rods	−	−			
KB06NF	−rods	−	+	EF589813 (49)	*Enterobacter* sp.	94%

3.3. Indole acetic acid production and phosphate solubilising properties

A colour change from yellow to red indicated the production of IAA and was recorded in four of the bacteria isolates — KB06NC, KB06G, KB06A and KB06D (Table 1). Isolate KB06NF was the only isolate that showed the capability to utilise the $CaHPO_3$ as indicated by an average clearing zone of 13 mm in diameter (Table 1).

3.4. Molecular identification and phylogenetic analyses

Bacterial isolates with significant stimulatory and inhibitory effects on the growth of the mycelium, IAA production and phosphate solubilising properties were selected for molecular identification. Selected isolates included KB06F1, KB06K, KB06NC, KB06ND KB06G, KB06L, and KB06NF (Table 1). The amplified DNA products were between 600 and 700 bp in length. The homology sequence and phylogenetical analyses of the 16S rDNA of the isolates indicated that they belong to three different clades that included Proteobacteria, Firmicutes and Actinobacteria (Fig. 2). Three of the isolates belonged to the Protebacteria clade that was subdivided into Alphaprotebacteria consisting of isolate KB06NC and Gammaproteobacteria consisting of isolates KB06G and KB06NF. The Actinobacteria cluster consists of only one isolate — KB06L, while the Firmicutes cluster consists of KB06K and KB06F1.

4. Discussion

In this paper, we presented the community of culturable bacteria associated with the ascocarps of *K. pfeilii*. Identification of bacteria inhabitants of the ascocarp could provide important information on the "yet to be described" life cycle of this fungus. Using established microbiological techniques, the screening process showed the presence of bacteria species from Firmicutes, Actinobacteria, Alphaproteobacteria and gammaproteobacteria represented by six genera namely — *Bacillus*, *Paenibacillus*, *Curtobacterium*, *Rhizobium*, *Enterobacter* and *Strenotrophomonas*. Among the isolates, *Bacillus* and *Rhizobium* have been previously reported as potential MHBs (Barbieri et al., 2000; Citterio et al., 2001).

The in vitro assay of the effect of the bacterial isolates on the growth of the fungus was conducted on FTN and nutrient poor media. The use of nutrient poor media was introduced in order to deprive the fungus of one or more essential nutrients, which subsequently allows for the observation of the aiding effects of the bacteria on the growth of the mycelium (Garbaye, 1994). Isolates with significant stimulatory effects on the FTN medium were KB06K (*Bacillus*) and KB06F1 (*Paenibacillus*). On the PFTN medium, its only isolate KB06F1 (*Paenibacillus*) that retained a significant stimulatory effect. This is probably the first report of *Paenibacillus* as potential MHB.

Isolates KB06K identified as a Gram positive close relative of *Bacillus* sp. produced a stimulatory effect on FTN medium. However, the stimulatory effect of this isolate could not be replicated on both WA and PFTN in spite the increased concentration of the isolate. This may result from the lack of adequate carbon in these media (Martinez-Romero et al., 1991). This outcome is contrary to the report by Sbrana et al. (2002) who isolated a *Bacillus* sp. from *Tuber borchii* which produced a significant stimulatory effect on the in vitro growth of the fungus on a modified Hagem–Modess agar medium. *Paenibacillus* spp. have been shown to be capable of degrading complex carbohydrates. For instance, the

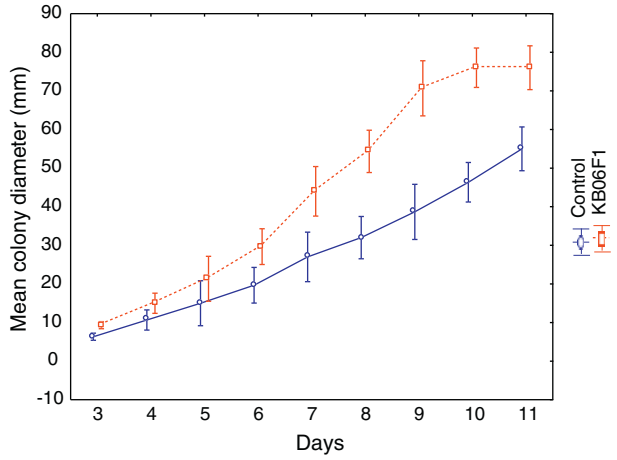

Fig. 1. Effect of bacterial isolate KB06F1 on the growth of *Kalaharituber pfeilii* as measured by colony diameter. $F_{(8,\ 32)} = 40.984$, $P < 0.05$; vertical bars denote 0.95 confidence intervals.

production of cellulolytic enzymes, such as xylanases and cellulases that degrade insoluble polysaccharides (cellulose and xylan) by *Paenibacillus curdlanolyticus* was reported by Pason et al. (2006). Additionally, some strains of the *Bacillus lautus* have been reported to produce dextran-degrading enzymes (Finnegan et al., 2004). These enzymes assist in softening the cell walls of the plant roots, thereby making fungal hyphal penetration easier to establish (Garbaye, 1994). In addition, *Paenibacillus* has been reported to occur in soil environments and in diesel sludge (Heyndrickx et al., 1996) where they participate in the degradation of complex carbohydrates (Heyndrickx et al., 1996; Finnegan et al., 2004). It can therefore be suggested that strain KB06F1 isolated in this study exhibited stimulatory effects on the growth of *K. pfeilii* mycelia through the degradation/hydrolysation of complex carbohydrates on the media thereby making them available in simpler and more absorbable forms such as glucose and sucrose (Fontana, 1968; Smith and Read, 2008). Such characteristics are very useful under natural conditions where complex sources of carbon can easily be made available for fungal nutrition. This result also revealed that the in vitro stimulatory effect produced by a *Paenibacillus* sp. on the growth of *K. pfeilii* is concentration dependent. This characteristic is consistent with what was reported by Aspray et al. (2006), where a strain of *Burkholderia* sp. EJP67 was found to be concentration dependent in its role as a MHB. Mycorrhizal formation was only stimulated at a concentration range of 10^7 to 10^9 CFU mL^{-1} in vitro. In contrast, Bending et al. (2002) reported a relationship between the mycorrhizal formation on *Pseudotsuga menziesii* (Douglas fir) and *Pseudomonas fluorescens* BBc6, where there was a decrease in mycorrhizal colonisation with increasing bacterial doses.

Isolates KB06NA, KB06NC and KB06ND did not produce any significant stimulatory effects on the growth of the fungal mycelia on the PFTN medium. Isolates KB06NC and KB06ND were identified with sequence homology and phylogenetic analysis as relatives of the *Rhizobium sp.* and *Strenotrophomonas* sp. respectively. Only isolate KB06NC (*Rhizobium* sp.) produced significant stimulatory effects on WA. Rhizobia are nitrogen fixing Gram negative rods that usually form symbiotic relationships with leguminous (Berkum et al., 1998) and non-leguminous (Antoun et al., 1998) plants. Close associations of *Rhizobium* with mycorrhizal plants have previously been described (Garbaye, 1994) to indirectly influence mycorrhizal formation. The isolation of a close relative of *Rhizobium* from the ascocarp harvested in this study could have originated from leguminous trees such as *Acacia erioloba* at the site of ascocarp collection (Taylor et al., 1995). The isolation of *Rhizobium* from *T. borchii* ascocarps was previously reported (Barbieri et al., 2000). Similarly, nitrogen fixation was confirmed by Barbieri et al. (2010) in *Tuber magnatum*. The significant stimulatory effect of *Rhizobium tropici* on the growth of *K. pfeilii* mycelium on WA could be attributed to their capability to supply the nitrogen requirement of the fungus, which is absent from the water agar. The FTN and PFTN media contained a nitrogen source which the fungus could access for growth. The positive effects of *Rhizobium* on mycorrhizal formation have been reported to be a result of improved plant health caused by the phytohormone production of this bacterium. However, the high demand for nitrogen by mycorrhizal fungi for the formation of chitin, the main constituent of their cell wall, is more likely the driving force behind the reliance of the fungus on the bacteria for nitrogen (Azcon-Agular and Barea, 1992; Xie et al., 1995).

Table 2
Mean growth of *Kalaharituber pfeilii* as measured by colony diameter on Fontana (FTN) medium, poor Fontana medium (PFTN) and water agar (WA) under the influence of different bacterial isolates after 11 days.

	FTN			PFTN			WA		
	$P < 0.05$, $F_{(136,\ 288)} = 53.449$			$P < 0.05$, $F_{(119,\ 252)} = 8.891$			$P < 0.05$, $F_{(153,\ 324)} = 15.705$		
Isolate	Colony diameter	Standard Error		Colony diameter	Standard Error		Colony diameter	Standard Error	
KB06A	50.3a	5.24	N	41.0a	3.06	N	16.0b	1.53	R
KB06C	53.7a	2.60	N	40.0a	1.15	N	19.0a	2.65	N
KB06D	29.3b	1.76	R	22.7b	1.45	R	21.7a	0.33	N
KB06E	40.3b	0.88	R	38.3a	1.76	N	23.7a	2.03	N
KB06F1	76.0c	0.00	S	41.0a	5.13	N	51.3a	8.82	N
KB06F2	42.3b	2.03	R	22.7b	3.84	R	16.7a	2.19	N
KB06G	46.3a	0.88	N	17.3b	7.42	R	14.0a	2.00	N
KB06H	52.3a	1.20	N	36.0a	2.08	N	47.3a	2.40	N
KB06I	38.0b	1.53	R	54.0a	1.53	N	40.3a	1.76	N
KB06J	36.0b	0.58	R	38.0a	7.09	N	38.7a	0.88	N
KB06K	68.0c	2.52	S	46.7a	2.33	N	32.7a	6.69	N
KB06L	27.0b	1.00	R	12.0b	3.06	R	33.0a	3.51	N
KB06NA	16.7b	1.20	R	59.3a	2.40	N	44.0a	3.79	N
KB06NC	39.3b	2.60	R	52.7a	2.19	N	76.0c	0.00	S
KB06ND	37.0b	0.58	R	55.7a	0.33	N	9.3b	0.67	R
KB06NE	25.7b	2.03	R	21.7b	5.84	R	25.7a	2.19	N
KB06NF	0.0b	0.00	I	24.0b	5.51	R	0.0b	0.00	I
Control	55.0a	2.89		45.3a	1.76	c	29.7a	0.88	

Different letters indicate significant differences at $p < 0.05$.
N = neutral; R = reduced growth; I = inhibitory; S = Stimulatory.

Table 3
Mean growth of *Kalaharituber pfeilii* as measured by colony diameter on water agar (WA) and poor Fontana medium (PFTN) under low (OD_{600} = 0.05) and high (OD_{600} = 0.5) concentrations of the bacterial isolate KB06F1.

Media	KB06F1 OD_{600}	Colony diameter		Standard error
WA	0.05	37.3a	N	0.88
WA	0.5	60.3b	S	3.18
Control (WA)		34.0a		2.52
PFTN	0.05	68.0a	N	6.56
PFTN	0.5	76.0b	S	0.00
Control (PFTN)		70.7a		5.33

Different letters indicate significant differences at $p < 0.05$
N = neutral; R = reduced growth; I = inhibitory; S = Stimulatory

Isolate KB06NF had a severe inhibitory effect on the mycelial growth. The isolate is a Gram negative rod and non-indole producer. Another isolate (KB06L) that had an inhibitory effect on the growth of the fungal mycelium was identified as *Curtobacterium* sp. This is a Gram positive, spore producing bacterium (Madigan et al., 1998). Although Barriuso et al. (2005) isolated a strain that was a phosphate solubiliser, this isolate did not compare. In addition, isolate KB06G identified as a close relative of *Stenotrophomonas* sp. produced inhibitory effects on the PFTN and FTN media. The large proportion of Gram positive (10 out of 17) bacteria isolated in this study agrees with the finding of Barbieri et al. (2005) where most of the bacteria associates of *T. borchii* were Gram positive. However, as the majority of these showed little or no effect on mycelial growth, they were not subjected to the identification process.

IAA production observed in four of the isolates indicated that they could be indirectly involved in mycorrhizal formation by improving the short root formation of the plants. However, only two of the isolates were identified, *Rhizobium* sp. and *Strenotrophomonas* sp. *Rhizobium sp.* exhibited potential to be a MHB by stimulating the growth of the fungus on WA. In contrast, *Strenotrophomonas* sp. was significantly inhibitory to the growth of the fungus, on both PFTN and WA media. The inability of this isolate to stimulate the growth of the fungus does not disqualify it from being a MHB. Positive effects on plant growth due to the high phytohormone production could indirectly influence mycorrhizal formation.

The bacterial isolate KB06NF (*Enterobacter* sp.) was the most inhibitory to the growth of the fungus and was the only phosphate solubilising bacteria isolated from the ascocarp of *K. pfeilii*. This could indicate that this bacterial isolate could contribute to the beneficial effects of mycorrhizal formation by solubilising the immobile phosphorus in the soil for eventual absorption by mycorrhizal fungi present in the soil. Such a process may improve the communication between the plant and the fungus, thereby encouraging early mycorrhizal formation (Garbaye, 1994).

The characteristics exhibited by isolates of KB06G and KB06NF raise concerns about the use of in vitro co-inoculation techniques to assess the MHB potential of bacteria as reported by Bowen and Theodorou (1979). Some bacteria that are neither stimulatory nor inhibitory to the growth of the fungus can still be classified as MHB, especially under natural conditions. It can therefore be suggested from this study that the ascocarp of *K. pfeilii* harbours many potential MHBs that may be capable of positively influencing mycelia growth and mycorrhizal formation both directly and indirectly. Furthermore, it is possible that the mycorrhizosphere of *K. pfeilii*/host plant may have a way of selectively influencing and structuring the microbial diversity of the region to encourage the survival of only those bacteria that are directly or indirectly beneficial to the mycorrhizal association or any of its partners (Frey-Klett and Garbaye, 2005; Frey-Klett et al., 2005).

It was difficult at this stage to confirm the inhibitory and stimulatory effects of these bacteria in vivo. Thus, caution must be exercised in extrapolating the type of result obtained in this study to the actual establishment of the mycorrhizal association as it has been shown that not all mycelial growth inhibition caused by bacteria in vitro prevents mycorrhizal establishment. Additional investigation into

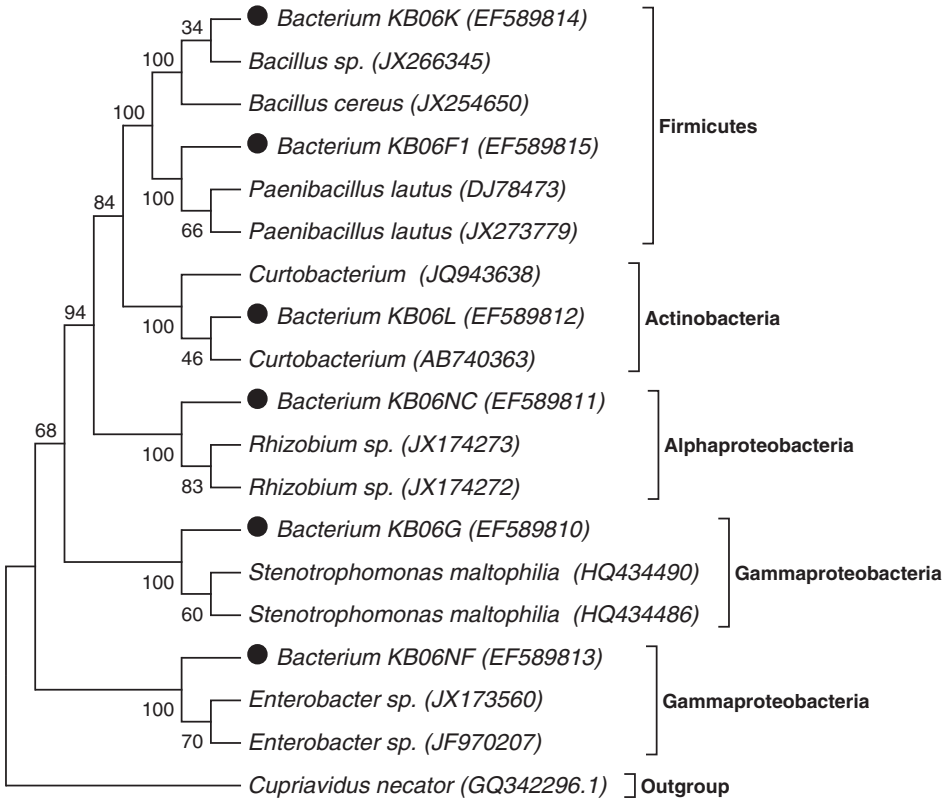

Fig. 2. Neighbour joining tree showing partial sequences of 16S rDNA of bacterial isolates obtained from the truffle ascocarp and their related species obtained from the GenBank as established by the bootstrap NJ method.

the tripartite association in vivo involving host plants, *K. pfeilii* and bacteria would provide more insights into the effects of the bacteria isolates on mycorrhizal formation, colonisation and subsequent beneficial effects on potential host plants.

This study has reported on the potential beneficial roles of bacteria associated with the fruiting bodies of *K. pfeilii* and has contributed to our understanding of the complexity involved in the biology of *K. pfeilii* and may therefore assist in the discovery of potential host plants of *K. pfeilii*. The emphasis was essentially on the culturable bacterial population associated with *K. pfeilii*. Considering that 95% of soil bacteria are yet to be cultured (Amann et al., 1995), there are likely to be many more unidentified bacterial associates. The use good molecular techniques such as next generation sequencing to identify possible genetic markers that are representative of MHB will go a long way to reducing the time and resources currently required for the investigation, identification and confirmation of these ecologically important bacteria. For example, Barbieri et al. (2005) used molecular techniques to detect some, yet-to-be cultured *Cytophaga–Flexibacter–Bacteroides* in ECM mycelium of *T. borchii*. These are soil associated bacteria that were recorded from truffles for the first time. Their roles in relation to the mycorrhizal formation are yet to be determined (Barbieri et al., 2005). Similarly, some fungal regulated genes were discovered in *Pseudomonas aeruginosa* by Hogan et al. (2004) during their investigation of the pathogenicity of the bacterium on the *Candida albicans* fungus. Such techniques could be applied to identify MHB from natural environments (Frey-Klett and Garbaye, 2005).

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References

Adeleke, R.A., 2007. Isolation, Propagation and Rapid Detection of Kalahari Truffle, a South African Mycorrhizal Fungus. Masters of Science Degree thesis Rhodes University, Grahamstown, South Africa.

Ahmad, F., Ahmad, I., Khan, M.S., 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turkish Journal of Biology 25, 29–34.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143–169.

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R., Lalande, R., 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). Plant and Soil 204, 57–67.

Aspray, T.J., Eirian Jones, E., Whipps, J.M., Bending, G.D., 2006. Importance of mycorrhization helper bacteria cell density and metabolite localization for the *Pinus sylvestris–Lactarius rufus* symbiosis. FEMS Microbiology Ecology 56, 25–33.

Azcon-Aguilar, C., Barea, J., 1992. Interactions Between Mycorrhizal Fungi and Other Rhizosphere Microorganisms. Chapman and Hall, Inc., New York, USA 163–198.

Barbieri, E., Potenza, L., Rossi, I., Sisti, D., Gimaro, G., Rossetti, S., Beimfohr, C., Stocchi, V., 2000. Phylogenetic characterization and in situ detection of a *Cytophaga–flexibacter–bacteroides* phylogroup bacterium in *Tuber borchii* vittad. ectomycorrhizal mycelium. Applied and Environmental Microbiology 66, 5035–5042.

Barbieri, E., Bertini, L., Rossi, I., Ceccaroli, P., Saltarelli, R., Guidi, C., Zambonelli, A., Stocchi, V., 2005. New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus *Tuber borchii* Vittad. FEMS Microbiology Letters 247, 23–35.

Barbieri, E., Ceccaroli, P., Saltarelli, Guidi, C., Potenza, L., Basaglia, M., Fontana, F., Baldan, E., Casell, S., Ryahi, O., Zambonelli, A., Stocchi, V., 2010. New evidence for nitrogen fixation within the Italian white truffle *Tuber magnatum*. Fungal Biology 114, 936–942.

Barriuso, J., Pereyra, M.T., Lucas, J.A., Megias, Gutierrez, M.J., Ramos, B., 2005. Screening for putative PGPR to improve establishment of the symbiosis *Lactarius deliciosus-pinus* sp. Microbial Ecology 50, 82–89.

Bending, G., Poole, E., Whipps, J., Read, D., 2002. Characterisation of bacteria from *Pinus sylvestris: Suillus luteus* mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiology Ecology 39, 219–227.

Berkum, V.P., Beyene, D., Bao, G., Campbell, T.A., Bertrand, D.E., 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) ledebour]. International Journal of Systematic Bacteriology 48, 13–22.

Bowen, G.D., Theodorou, C., 1979. Interactions between bacteria and ectomycorrhizal fungi. Soil Biology and Biochemistry 11, 119–126.

Citterio, B., Malatesta, M., Battistelli, S., Marcheggiani, F., Baffone, W., Saltarelli, R., Stocchi, V., Gazzanelli, G., 2001. Possible involvement of *Pseudomonas fluorescens* and Bacillaceae in structural modifications of *Tuber borchii* fruit bodies. Canadian Journal of Microbiology 47, 264–268.

Ferdman, Y., Aviram, S., Roth-Bejerano, N., Trappe, J.M., Kagan-Zur, V., 2005. Phylogenetic studies of *Terfezia pfeilii* and *Choiromyces echinulatus* (pezizales) support new genera for southern African truffles: *Kalaharituber* and *Eremiomyces*. Microbiological Research 109, 237–245.

Finnegan, P.M., Brumbley, S.M., O'Shea, M.G., Nevalainen, K.M.H., Bergquist, P.L., 2004. *Paenibacillus* isolates possess diverse dextran-degrading enzymes. Journal of Applied Microbiology 97, 477–485.

Fontana, A., 1968. Miceli di funghi ipogei in coltura pura. Estratto Delgi Atti del Congresso Internazionale sul Tartufo, Spoleto. Maggio, pp. 24–25.

Frey-Klett, P., Garbaye, J., 2005. Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal-bacterial interactions. New Phytologist 168, 4–8.

Frey-Klett, P., Chavatte, M., Clausse, M.L., Courrier, S., Le Roux, C., Raaijmakers, J., Martinotti, M.G., Pierrat, J.C., Garbaye, J., 2005. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytologist 165, 317–328.

Garbaye, J., 1994. Tansley review no. 76. Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytologist 128, 197–210.

Gordon, S.A., Weber, R.P., 1951. Colorimetric estimation of indole acetic acid. Plant Physiology 26, 192–195.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symposium Series 41, 95–98.

Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Kersters, K., de Vos, P., Logan, N.A., Aziz, A.M., Ali, N., Berkeley, R.C., 1996. A polyphasic reassessment of the genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and of *Bacillus peoriae* (Montefusco et al. 1993) as *Paenibacillus peoriae* comb. nov., and emended descriptions of *P. lautus* and of *P. peoriae*. International Journal of Systematic Bacteriology 46, 988–1003.

Hogan, D.A., Vik, A., Kolter, R., 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. Molecular Microbiology 54, 1212–1223.

Kagan-Zur, V., Kuang, J., Tabak, S., Taylor, F.W., Roth-Bejerano, N., 1999. Potential verification of a host plant for the desert truffle *Terfezia pfeilii* by molecular methods. Mycological Research 103, 1270–1274.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30, 3059–3066.

Madigan, M.T., Martinko, J.M., Parker, J., 1998. Brock Biology of Microorganisms. Prentice-Hall International, USA.

Martinez-Romero, E., Segovia, L., Mercante, F., Franco, A., Graham, P., Pardo, M., 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. International Journal of Systematic Bacteriology 41, 417–426.

Mehta, S., Nautiyal, C.S., 2001. An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology 43, 51–56.

Molina, R., Palmer, J.G., 1982. Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck, N.C. (Ed.), Methods and Principles of Mycorrhizal Research. The American Phytopathological Society, USA, pp. 115–129.

Moore, D.D., Ausubel, F.M., Kingston, R., Smith, J.A., Brent, R., 1987. Current Protocols in Molecular Biology. Wiley, John and Sons, Incorporated, USA.

Myers, R.M., Fischer, S.G., Lerman, L.S., Maniatis, T., 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Research 13, 3131–3145.

Pason, P., Kyu, K.L., Ratanakhanokchai, K., 2006. *Paenibacillus curdlanolyticus* strain B-6 xylanolytic–cellulolytic enzyme system that degrades insoluble polysaccharides. Applied and Environmental Microbiology 72, 2483–2490.

Percudani, R., Trevisi, A., Zambonelli, A., Ottonello, S., 1999. Molecular phylogeny of truffles (Pezizales: Terfeziaceae, Tuberaceae) derived from nuclear rDNA sequence analysis. Molecular Phylogenetics and Evolution 13, 169–180.

Poole, E.J., Bending, G.D., Whipps, J.M., Read, D.J., 2001. Bacteria associated with *Pinus sylvestris–Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytologist 151, 743–751.

Sbrana, C., Agnolucci, M., Bedini, S., Lepera, A., Toffanin, A., Giovannetti, M., Nuti, M.P., 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth. FEMS Microbiology Letters 211, 195–201.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis. Academic Press, Oval Road, London NW1 7DX, UK 24–28.

StatSoft, I., 2005. STATISTICA (data analysis software system). version 7.1. www.statsoft.com.

Suresh, C.K., Bagyaraj, D.J., 2002. Mycorrhiza-microbe interface: effect on rhizosphere. In: Sharma, A.K., Johri, B.N. (Eds.), Arbuscular Mycorrhizae. Scientific Publishers, Enfield, New Hampshire, USA, pp. 7–28.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software. 4.0.

Taylor, A.F., Alexander, I., 2005. The ectomycorrhizal symbiosis: life in the real world. Mycologist 19, 102–112.

Taylor, F.W., Thamage, D.M., Baker, N., Roth-Bejerano, N., Kagan-Zur, V., 1995. Notes on the Kalahari desert truffle, *Terfezia pfeilii*. Microbiology Research 99, 874–878.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., Dicuccio, M., Edgar, R., Federhen, S., Geer, L.Y., Kapustin, Y., Khovayko, O., Landsman, D., Lipman, D.J., Madden, T.L., Maglott, D.R., Ostell, J., Miller, V., Pruitt, K.D., Schuler, G.D., Sequeira, E., Sherry, S.T., Sirotkin, K., Souvorov, A., Starchenko, G., Tatusov, R.L., Tatusova, T.A., Wagner, L., Yaschenko, E., 2006. Database resources of the national center for biotechnology information. Nucleic Acids Research 34 (Database issue D173-D180_([online]. Available from: www.ncbi.nlm.nih.gov.).

Xie, Z.P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W.J., Vogeli-Lange, R., Boller, T., 1995. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiology 108, 1519–1525.