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Molecular characterization of some bacteria and fungi associated with the decomposition of leaf litters of *Eucalyptus camaldulensis* and *Tectona grandis*

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Abstract

***Eucalyptus camaldulensis* and *Tectona grandis,* have the characteristics of fast growth rate. The decomposition of litter biomass of these exotic plants is necessary in order to return the nutrients taken up by the plants. Some of the major nutrients include nitrogen, carbon, phosphorus, potassium, calcium and magnesium. To have a better understanding of the decomposition of leaf litters of *E. camaldulensis* and *T. grandis,* bacteria and fungi associated with their decomposition were investigated. Soil and leaf litters of these exotic plant species were collected from six sampling sites of each plantation designated as E1 – E6 for *E. camaldulensis* and T1 – T6 for** T. grandis, **respectively. Litterbag technique was employed for the decomposition study which lasted for 60 days. Using standard methods, analysis of carbon (IV) oxide evolution and microbial population were done at day 1, 30 and 60 of the decomposition period. Results showed that there was a general decrease in the percentage mass loss of** E. camaldulensis **and** T. grandis **leaf litter samples, which ranged from 71-85% and 40-61% respectively. The amount of carbon, nitrogen, phosphorus, potassium, calcium and magnesium decreased in the leaf litter but increased in the soil during decomposition. The evolution of CO_2 was highest in E4 on day 60 (554.4 μg/g/day) among** E. camaldulensis **samples, whereas the evolution of CO_2 was highest in T2 on day 30 with a value of 516.5 μg/g/day among *T. grandis* samples. The bacteria isolated during the decomposition periods were *Bacillus licheniformis, Pseudomonas putida, Bacillus subtilis, Micrococcus luteus, Proteus vulgaris* while fungi were *Aspergillus niger* and *Penicillium notatum. Bacillus licheniformis* was the most prevalent bacteria isolated. There is need to molecularly establish the specific capability of each microbial isolate with the view to identifying those that sequentially elaborate enzymes that can completely metabolize the leaf litters to their elemental form for adequate enrichment of the soil. This will enhance rapid recovery of the composite undergrowth and also promote diverse microbial colonization.**

Keywords: Decomposition, leaf litters, *Eucalyptus camaldulensis*, bacteria, fungi, and *Tectona grandis.*

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Introduction

Microorganisms such as bacteria, archaea, fungi, and protozoans, are very important in all processes related to decomposition of leaf litter. The microbial components of soil are entirely responsible for the decomposition of organic matter (Forsyth, 2009). The roles of microbes as summarized by Hoff et al. (2004) include

extracellular and intracellular decomposition of complex nutrient sources, transportation of simple nutrients across cell membranes for metabolic processes and detoxification of compounds that could inhibit microbial growth. Statmets (2005) reported that microbial decomposition of leaf litters allows carbon, nitrogen, phosphorus, potassium, calcium, zinc and other minerals to be deposited back into the nutritional bank. The findings of Rigbelis and Nahas (2004) revealed that microbial leaf litter results the release of nutrients to the soil in the forest environment.

Soil being one of the most complex and heterogeneous environments, contains significant microbial diversity that plays major role in decomposition (Tiedje et al., 1999). The microbiology of soil has been studied and many novel strains associated with decomposition have been isolated (Sait et al., 2002). Rigobelo and Nahas (2004) stated that among the soil organisms, bacteria and fungi present the highest values of biomass and respiratory metabolism, and participate actively in litter decomposition process; bacteria which represent the major group are responsible for 25 to 30% of the total soil microbial biomass. Extensive studies on microbial diversity of soils and the leaf litter biosphere, have suggested that members of the phyla Actinobacteria and Proteobacteria dominate these environments (Xu et al., 1996; Sharma et al., 2005).

Eucalyptus camaldulensis and *Tectona grandis* are trees that grow extremely fast in warm and humid climates. These plants are most widely planted because of their adaptation to their environment (Jairus et al., 2011). According to reports of some studies, the leaf litter of the exotic plants contain at least eight kinds of chemical substance, such as alkanes, aromatic hydrocarbons, esters etc (Goya et al., 2008). Among these compounds, the phenols, terpene and esters are the major allelochemicals, which can influence the growth of undergrowth (Yu et al., 2009) and therefore, microbial diversity and activities would be affected by leaf chemical composition. Litter study carried out by Meentemeyer (1978) showed that carbon, nitrogen and lignin released from plant material influence and control the rates of decomposition. Nutrients released from microbial decomposition of plant materials, such as leaf litter, in both natural and agricultural ecosystems, are important for plant growth. It is well known that the mass of leaf litter decreases during the process of decomposition due to the loss of readily available carbon and other nutrients; microbial population increases likewise (Wardle et al., 2006).

These exotic plant species presumptively take up nutrients at a fast rate, hence their rapid growth. Rapid nutrient uptake could lead to loss of soil nutrient if replenishment is inadequate. In the light of this, need arises for a rapid decomposition of these exotic plants by the action of microorganisms to balance the quick uptake of nutrient from the soil, hence, prevent degradation of soil. Soil microbial biomass, as documented by Sicardi et al. (2004), is suitable indicator for the measurement of organic matter in the soil. Microorganisms are also responsible for the mineralization process in plantation ecosystem. To understand the mechanisms underlying nutrient cycling, it is paramount to identify the key parameters such as decomposition of leaf litters, nutrient release from the decomposing leaf litters and microorganisms associated with the decomposition. A detailed study of the microorganisms responsible for the rapid decomposition and mineralization of plant leaf litters is therefore important to evaluate whether nutrient cycling under these plantations can be facilitated artificially, by genetically enhancing less efficient decomposing and/or deliberating introducing more efficient microbes.

The objective of this present study was to isolate and characterize microorganisms, which are associated with the decomposition of leaf litters of *E. camaldulensis* and *T. grandis.* Evaluation and comparison of microbial population and types was carried out, to determine any successional changes in the numerical and biodiversity of microbes during the decomposition of organic matter. The study was limited to the investigation of bacteria and fungi as they present the highest biomass and respiratory mechanism (Rigobelis and Nahas, 2004), thus having greater contribution to organic matter decomposition processes. The amount of carbon (IV) oxide evolved during decomposition were measured and it is an index of microbial activity (Mary and Sankaran, 1991).

Materials and Methods

Study site

This study was conducted in two plantations in Afaka, Kaduna State, Nigeria. The coordinates of the study area are latitude 10° 36' 21.28" N and longitude 7 ° 18' 57.17" E, with mean annual rainfall of about 1200 mm in the rainy season and the mean annual temperature of about 24.5°C (Nwaedozie et al., 2013).

Sample collection and Decomposition assay

Using a table of random number, soil samples as well as leaf litter samples were collected randomly from six sampling sites of each plantation (*E. camaldulensis* and *T. grandis* plantations). The litter decomposition study was carried out using the mesh bag technique described by Mary and Sankaran (1991). Ten grams (10 g) of air-dried leaf litter of each exotic plant species as well as its corresponding soil from a sampling site were transferred separately in nylon mesh bags- (mesh size 2 mm) and closed firmly. A total of 18 litter bags were used for each plant species. The soil and its litter samples obtained from a particular sampling site were triplicated to facilitate their analyses in terms of mass loss and quantification of carbon (IV) oxide evolved. The litter and soil was watered with 20 ml of distilled water periodically to maintain 60-70% water holding capacity. Control sets contained soils without leaf litters.

Respiration rate was determined by measuring and quantifying the evolution of CO_2 from the decomposing litter according to the method of Qingkui et al. (2008). Glass vials containing 10 ml of 0.5 M sodium hydroxide (NaOH) to trap the evolved CO_2 were incorporated into each litter bag. After 3 days of introducing the NaOH, the glass vials were removed and the CO_2 trapped in NaOH was determined by titrimetry. The amount of CO_2 evolved was expressed in µg CO_2 g^{-1} oven dry litter day^{-1}. The determination of CO_2 evolution was carried out at monthly intervals. Difference in the evolution of Carbon (IV) oxide at day 1, 30 and 60, was compared using One-way Analysis of Variance.

Isolation of bacteria and fungi

Isolation of microbial isolates of the samples was limited to predominant bacteria and fungi. The standard method as described by Alexander (1982) was adopted for the determination of the total number of viable bacteria and fungi. One gram of the decomposing litter was suspended in 10 ml of sterilized water and five-fold serial dilutions of the suspension were prepared for accurate counting of the bacteria and fungi. For determination of total viable bacteria and fungi, nutrient and potatoes dextrose agar media with inoculated with the five-fold serial dilutions of the suspension. Isolation of bacteria and fungi was carried out at day 1, 30 and 60 of the decomposition period. The choice of interest was based on the frequency of occurrence. Characteristics of the isolates were compared with the properties described in Bergey's Manual of Systematic Bacteriology. Biochemical tests were also carried out to identify the organisms, which include starch hydrolysis, urease, catalase, dehydrogenase, oxidase, methyl red, nitrate reduction, indole, voges-proskauer, H_2S production and sugar fermentation test (Alexander, 1977).

Extraction of genomic DNA for characterization of bacterial isolates

The DNA of the bacterial isolates was extracted using phenol/chloroform method (Zhang et al., 2007). A volume of 1.5ml of the bacterial cells was added to a micro-centrifuge tube and centrifuged for 30 seconds at 8000 rpm at 4°C. The cell pellets were suspended in 500 µl Tris-EDTA buffer and 350µl of 2% sodium dodecyl sulphate (SDS) was added. RNase of 50 µl was added into the tube and incubated at 37°C for 15 minutes. Pronase of 35 µl was also added and heated at 50°C until lysis completely took place. An equal volume of phenol/chloroform (700µl) mixture was then added into the micro-centrifuge tube and centrifuged at 10000rpm at 4°C for 5 minutes. The aqueous DNA layer was pipetted; DNA was precipitated using sodium acetate and isopropanol. One ml of 70% ethanol was added to wash the DNA. The DNA was dried at room temperature and dissolved in 50 µl Tris-EDTA (TE) buffer. The DNA concentration was determined by measuring the absorbance at 260nm, taking 1 absorbance unit as equal 50 µg/ml of double stranded DNA (Ausubel et al, 1994). The purity of the DNA preparation was determined by calculating the ratio of the absorbance at 280nm to 260nm, a ratio of 1.8 or above indicated a pure sample of DNA.

Amplification of 16S rDNA by Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification was carried out as performed by Thompson et al. (1994) using DNA taq polymerase. The isolated DNA was amplified using 16S rDNA universal primers (Forward, GGACTACAGGGTATCTAAT) and (reverse, AGAGTTTGATCCTGG).The PCR conditions were 95°C for 2 min to denature the template, followed by varied temperatures of 55-62°C for 30sec (primer annealing) and 72°C for 1-4 min (extension). A final cycle at 72°C for 10 min was used for extension. Precise conditions of annealing temperature and extension times were determined based on the primer melting temperature (T_m) and fragment length.

Nucleotide sequencing and alignment

DNA fragments were separated by agarose gel electrophoresis using 1 % w/v agarose in Tris-acetate-EDTA (TAE) buffer (Sambrook et al., 1989). Automated DNA sequencing was performed. Sequences were compared to other sequences in the GenBank databases using the Basic Local Alignment Search Tool (BLAST) package at http://www.ncbi.nlm.nih.gov/blast/ (Altschul et al., 1990) and Clustal X were used for 16S rDNA sequence alignments. For phylogenetic tree construction, multiple sequences were obtained from GenBank and the alignments were performed using MEGA6 (Thompson et al, 1994).

Results

Mass loss and Carbon (IV) oxide evolution from the decomposing leaf litter samples

There was a general sharp decline in the mass of all the leaf litters with increase in duration of decomposition time (Fig. 1 and 2). At day 60, the percentage mass loss of *E. camaldulensis* and *T. grandis* leaf litters ranged from 71-85% and 40-61% respectively (Fig. 1 and 2). As depicted in Fig. 3, the amount of carbon, nitrogen, phosphorus, potassium, calcium and magnesium decreased in the leaf litters but increased in the soil. The Carbon (IV) oxide evolved was more in *E. camaldulensis* and *T. grandis* leaf litters than the control (Fig. 4). The amount of CO_2 evolved was highest in E4 on day 60 (554.4 µg/g/day) in comparison with other samples of *E. camaldulensis* leaf litter samples (Fig. 4). On day 60, T2 evolved the highest amount of CO_2 with a value of 516.5 µg/g/day relative to other *T. grandis* litter samples while T4 evolved the least amount of CO_2 with a value of 156.7 µg/g/day (Fig. 4).

Total viable bacteria and diversity

Except for *T. grandis* at day 30 and 60 for samples 2 and 6, the population of total viable bacteria apparently increased marginally throughout the period of decomposition in all the samples (Table 1). There was no significant difference in the number of bacteria in the samples during decomposition as determined by ANOVA, having a P-Value of 0.270 for *E. camaldulensis* samples and 0.515 for *T. grandis* (Table 3).

The bacteria isolated from the decomposing leaf litters of *T. grandis* and *E. camaldulensis* at different periods of decomposition, except for *Pseudomonas putida* and *Proteus vulgaris,* were predominantly rod-shaped gram positive. The others were identified as *Bacillus licheniformis, Bacillus subtilis* and *Micrococcus luteus* (Table 4). In *T. grandis* samples, *Pseudomonas putida* was isolated on day 1, 30 and 60 while *Bacillus subtilis* was isolated on day 30 and 60. *Bacillus licheniformis* and Micrococcus luteus were only isolated on day 1 whereas, *Proteus vulgaris* was only isolated on day 60. In *E. camaldulensis* samples, *Bacillus licheniformis* was isolated on day 1, 30 and 60. *Micrococcus luteus* and *Proteus vulgaris* were isolated on day 1 and 30. *Pseudomonas putida* was isolated only on day 30 (Table 4). The phylogenetic position of *Bacillus licheniformis* which is the most prevalent bacteria isolated from both leaf litters is 99% similar to the *Bacillus licheniformis* 9945A in the GenBank (Fig. 5).

Total viable fungi and diversity

The fungal count of day 60 universally increased above those of day 1 for both plant litters' decomposition in all six samples (Table 2). Result of ANOVA shows that the population of fungi in both samples of *E. camaldulensis* and *T. grandis* are not significantly different, with a P-Value of 0.422 and 0.38 respectively (Table 3). The fungi isolated from both tree species were morphologically identified as *Aspergillus niger* and *Penicillium notatum*.

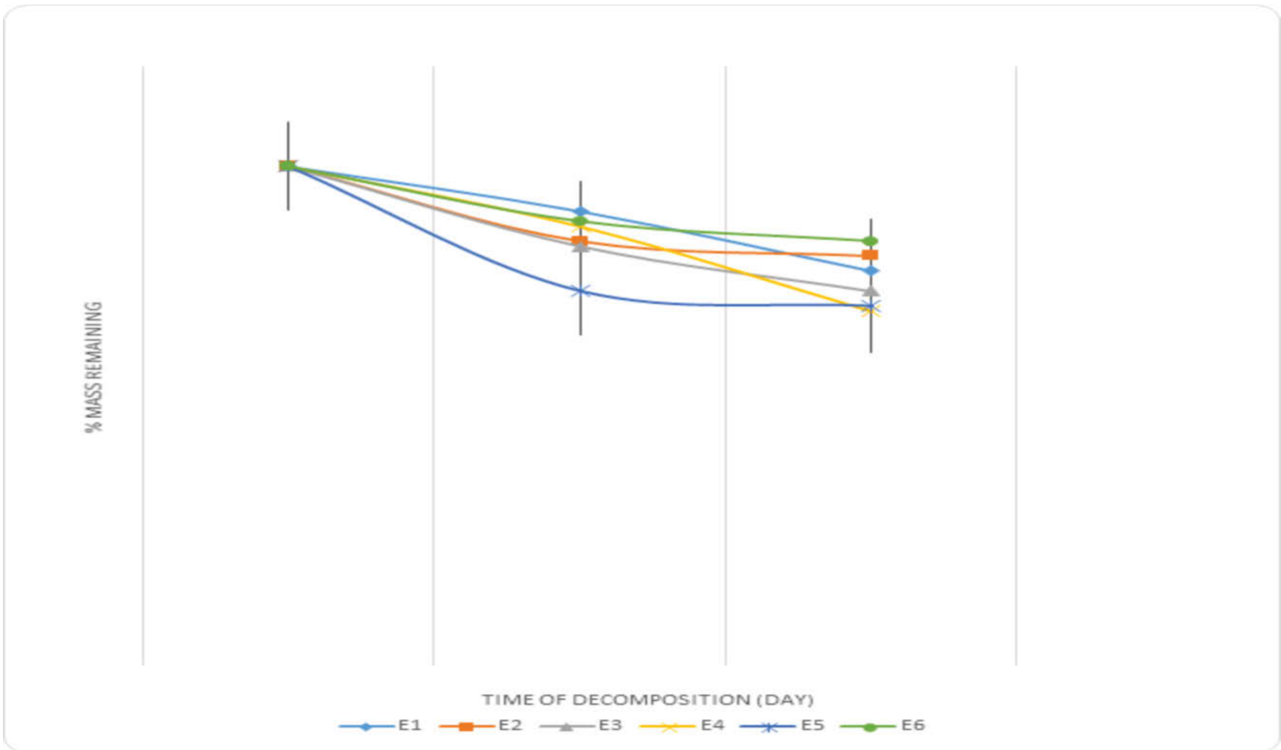

Fig. 1: Percentage mass of *E. camaldulensis* leaf litter remaining after different periods of decomposition by bacteria and fungi. E1 – E6 are sample 1 to 6.

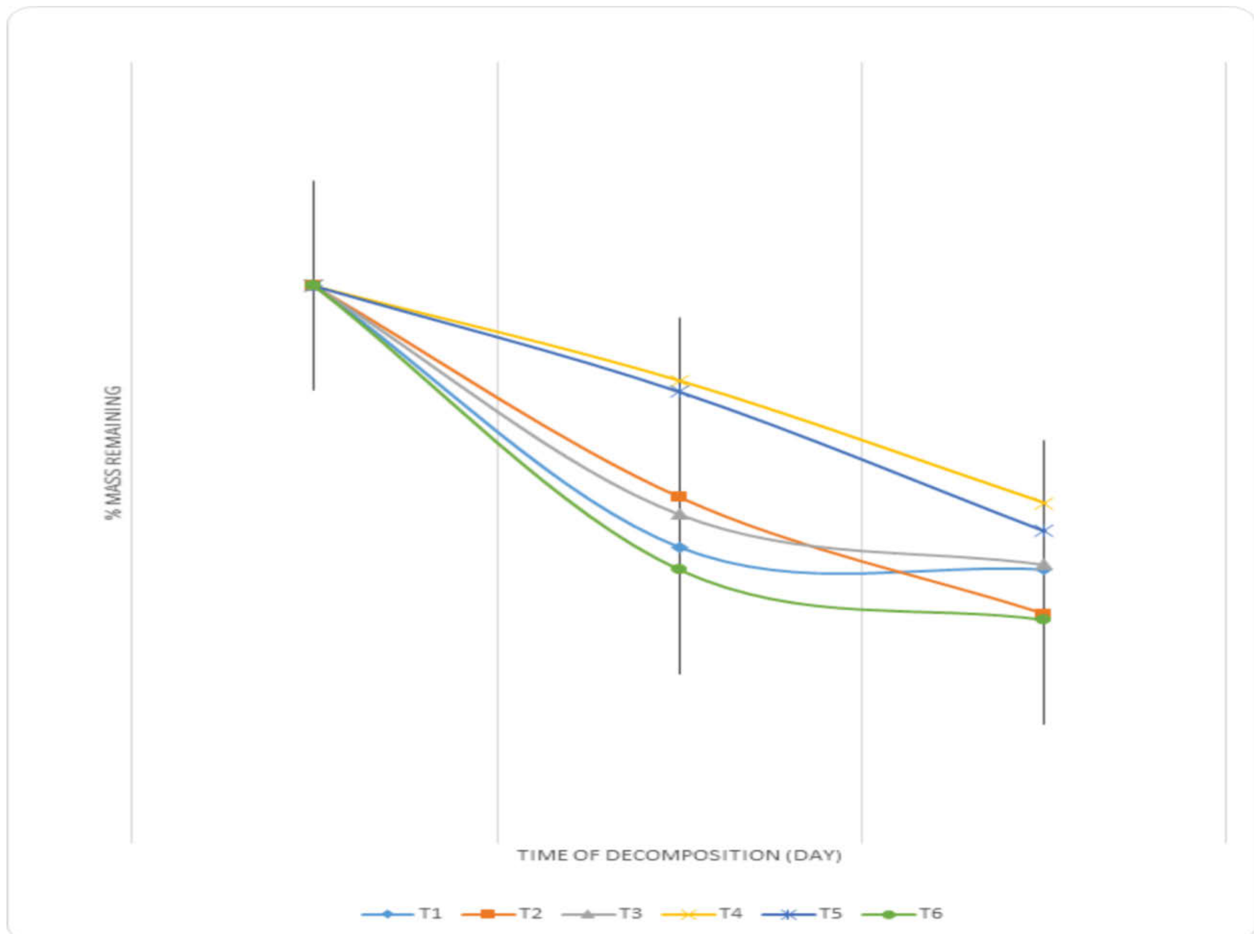

Fig. 2: Percentage mass of *T. grandis* leaf litter remaining after different periods of decomposition by bacteria and fungi. T1 – T6 are sample 1 to 6.

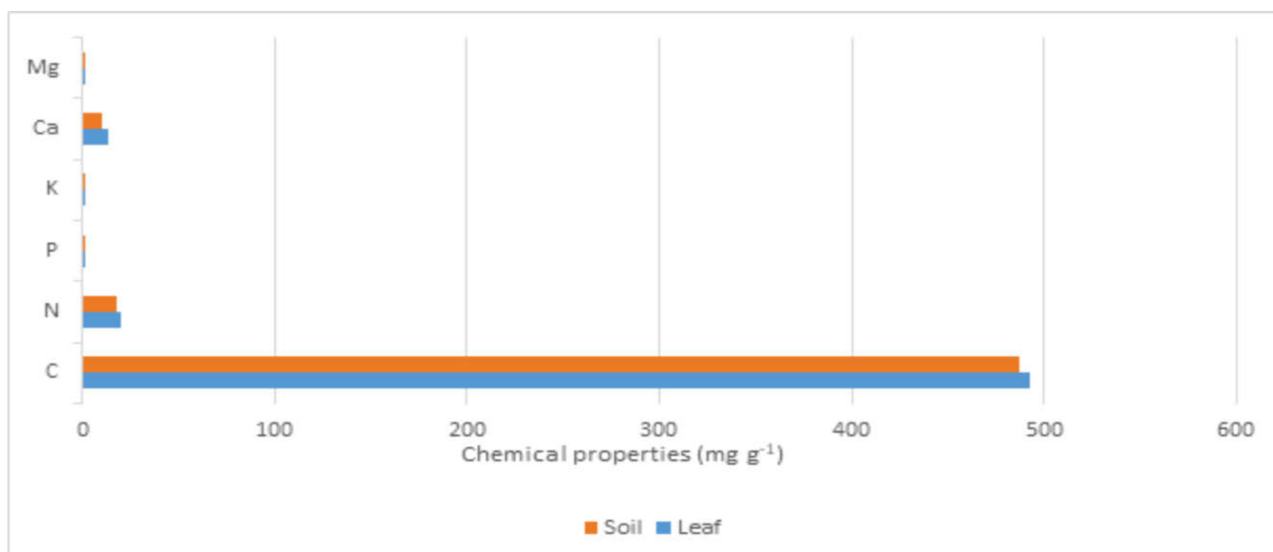

Fig. 3. Average chemical properties of leaf litter and soil of *E. camaldulensis* samples

Table 1: Population of total viable bacteria (CFU/ ml) $\times 10^6$ during the decomposition of leaf litter of *E. camaldulensis* and *Tectona grandis*

Samples	Day 1		Day 30		Day 60	
	E. camaldulensis	T. grandis	E. camaldulensis	T. grandis	E. camaldulensis	T. grandis
1	11	21	13	22	19	25
2	17	22	17	24	18	23
3	9	15	12	18	15	22
4	12	15	15	20	18	24
5	15	19	16	21	19	23
6	10	17	14	26	16	20

Table 2: Population of total viable fungi (CFU/ ml) $\times 10^3$ during the decomposition of leaf litter of E. camaldulensis and Tectona grandis

Samples	Day 1		Day 30		Day 60	
	E. camaldulensis	T. grandis	E. camaldulensis	T. grandis	E. camaldulensis	T. grandis
1	18	17	19	20	20	26
2	19	18	17	15	19	20
3	18	11	17	17	17	18
4	20	19	19	18	16	21
5	15	14	18	19	24	26
6	21	22	20	22	23	21

Table 3: Analysis of variance of the total viable bacteria and fungi during leaf litter decomposition of *E. camaldulensis* and *T. grandis*

	Bacteria		Fungi	
	E. camaldulensis	T. grandis	E. camaldulensis	T. grandis
F-value	1.47	0.849	1.07	1.20
P-value	0.270	0.515	0.422	0.38

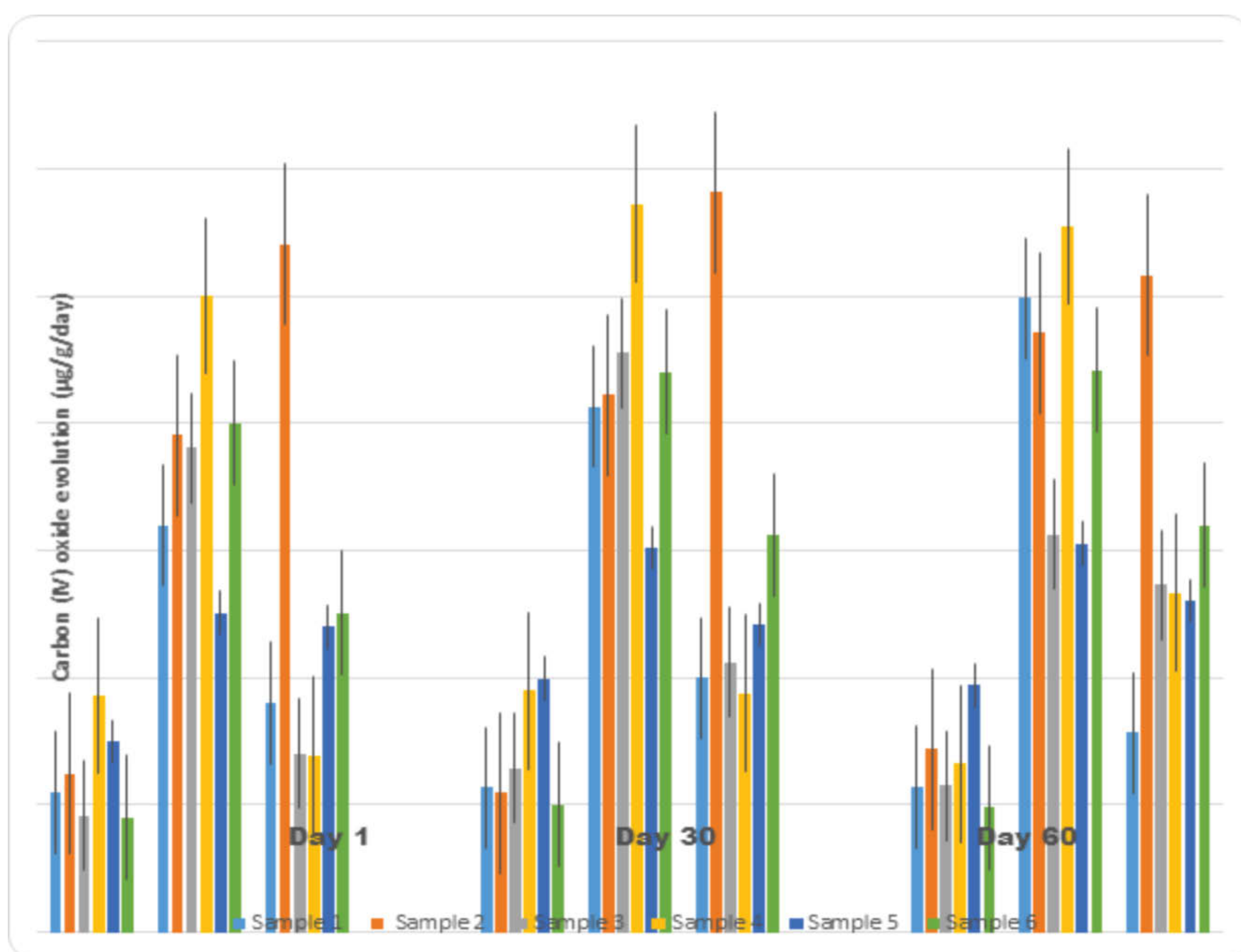

Fig. 4. Amount of carbon (IV) oxide evolved during the decomposition of *E. camaldulensis* and *T. grandis* by bacteria and fungi

Table 4: Morphological and Biochemical Characteristics of Bacteria isolated from *Eucalyptus camaldulensis* and *Tectona grandis* leaf litters at different period of decomposition.

Phenotypic and biochemical Characteristics		Bacteria Isolates							
		Day 1			Day 30			Day 60	
		A	B	C	A	B	C	A	B
Colony shape		Rod	Rod	Cocci	Rod	Rod	Cocci	Rod	Rod
Colony colour		Cream	Creamy	Yellow	Creamy	Creamy	White	White	Creamy
Opacity		Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Motility		+	+	-	+	+	-	+	+
Gram's staining		+	-	+	+	-	+	+	-
Starch hydrolysis		+	+	-	+	-	-	+	+
Urease test		+	-	-	+	-	-	+	-
Catalase test		+	+	+	+	+	+	+	+
Dehydrogenase		+	-	-	+	-	-	+	+
Oxidase		+	+	-	+	+	-	+	+
Methyl red		+	-	+	+	-	+	-	-
Nitrate reduction		+	+	+	+	+	+	+	+
Indole test		+	+	-	-	-	+	+	+
Voges-Proskauer		+	+	-	+	-	-	-	+
Sugar fermentation	Glucose	++	+-	+-	++	+-	+-	+-	++
	Fructose	++	++	+-	++	+-	++	+-	++
	Mannitol	+-	-	+-	++	-	+-	++	-
	Lactose	-	++	-	+-	-	-	+-	+-
	Sucrose	++	++	+-	++	+-	+-	++	++
	Maltose	+-	++	+-	++	+-	+-	++	+-
H_2S production		+	+	-	-	-	-	+	+
Probable genera		Bacillus licheniformis*	Proteus vulgaris	Micrococcus luteus*	Bacillus licheniformis	Pseudomonas putida*	Micrococcus luteus	Bacillus licheniformis	Proteus vulgaris*

Key: + Positive, - Negative, ++Acid and gas production, +- Acid production only. *Bacteria isolated in both leaf litter samples

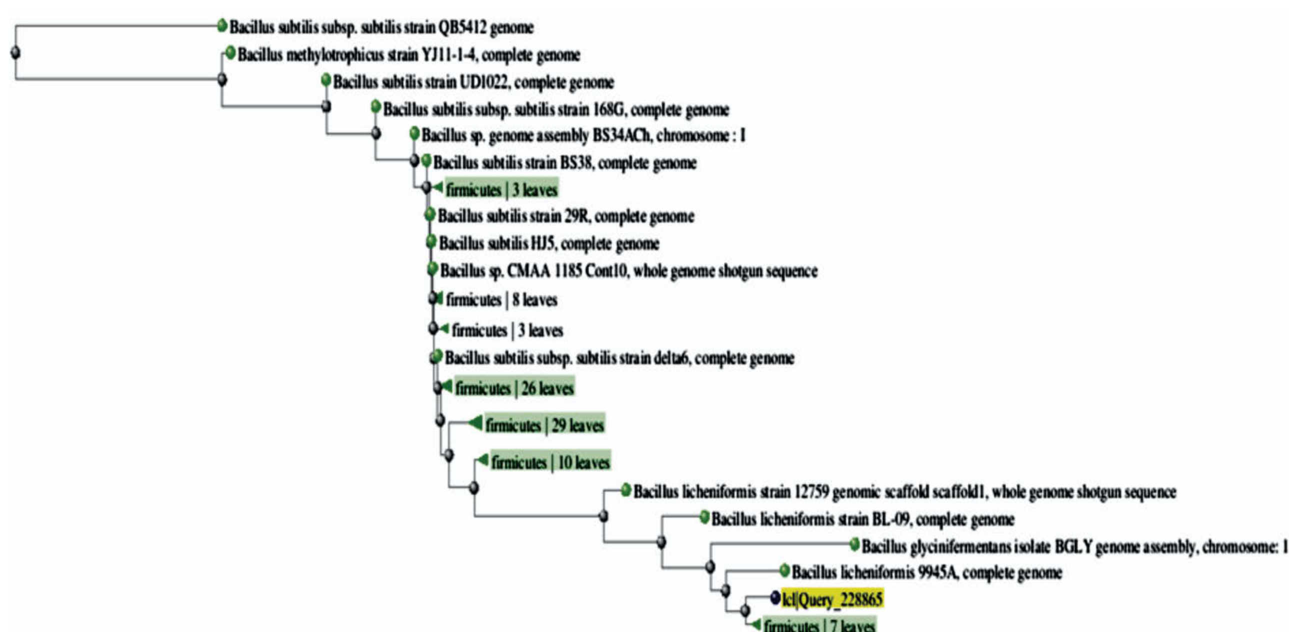

Fig. 5: Phylogenetic position of *Bacillus licheniformis* (Query sequence) within the radiation of members of the roundspore-forming, family *Planococcaceae*, order *Bacillales*, phylum *Firmicutes.* 99% similarity with *B. licheniformis*

Discussion

Microbial activity in terms of the quantity of carbon (IV) oxide evolved, was different in all the samples of both plant species. This suggests that varied nutrient availability of plant leaf litter and site conditions might have affected microbial activities. Higher CO_2 evolution on day 60 of decomposition in all the samples could imply intense microbial activities with longer duration of decomposition. This finding agrees with Rejmankova and Sirova (2007) who reported that microbial activities are high at the later stage of decomposition when litters have high availability of nutrient. Decomposition was more at the later stage because of concomitant increase in the population of microbes. Also, variation in microbial activity between litters of all six sites may be attributed to the differences in the physicochemical properties of the litters, which directly influence the rate of decomposition (Swift et al., 1979).

Microbial population is dependent on the rate of decomposition of organic matter. The number of microorganisms isolated during the decomposition were more in *T. grandis* than in *E. camaldulensis*. The findings of this study is in consonance with the findings of Behera and Sahani (2003) who suggested that compounds from the leaves of *Eucalyptus camaldulensis* might have had toxic effects on soil microorganisms. The population of viable bacteria and fungi were higher in *T. grandis* litter samples than in *E. camaldulensis* (Tables 1 and 2) as per the observation of Adekunle and Dafiwhare (2011). This result is in conformity with the findings of Singh (1969), who reported that the low population of total viable microbes in *Eucalyptus* litter samples in comparison to those of *Albizia* spp and *Tectona* spp, might be due to the presence of polyphenols in Eucalyptus spp leaves. Reduced microbial activity and population may also be attributed to the hard texture of the leaves.

Allelopathic compounds such as ester, terpene, phenols and tannin, produced from Eucalyptus spp. during its decomposition affect the population of soil microbes (Goya et al., 2008; Yu et al., 2009). However, the observable increase in soil microbial population at the later period of decomposition has negative consequences on the degradation of leaf litters (Steinbeiss et al., 2008; De Deyn et al., 2009, Malik et al., 2013). The population of bacteria were more than fungi in all the sites. This supports the claim of Alexander (1977) that bacteria are by far the most abundant group of soil microbes in term of number. Rigobelis and Nahas (2004) reported that bacteria are the major group of microorganisms responsible for 25-30% of the total soil microbial biomass.

Bacillus licheniformis, Bacillus subtilis, Pseudomonas putida, Micrococcus luteus and *Proteus vulgaris*, were common to both tree decomposing leaf litters. Adekunle and Dafiwhare (2011) opined that these bacteria are "soil habitat generalists". *Bacillus* spp. were the most abundant bacteria isolated from *E. camaldulensis* and *T. grandis* at different periods of decomposition. This corroborates the findings of Bigelow et al (2004) and Rigbelis and Nahas (2004), who reported that *Bacillus* spp. are able to survive adverse environmental conditions by producing extremely drought resistant endospores and they could also thrive under any type of vegetation. *Aspergillus niger* and *Penicillium* spp were the commonest genera of fungi isolated from the decomposing leaf litters of *E. camaldulensis* and *T. grandis;* This is in agreement with Kshattriya (1992), who reported three species of *Aspergillus* spp in the leaf litter decomposition in Sal forests of Central India. The presence of *A. niger* and *Penicillium* spp can be attributed to their ubiquitous nature, their ability to grow over a wide range of environmental conditions and their ability to degrade organic polymers. The decay study of leaf litter of Teak and Eucalyptus conducted by Soni (1985) showed the presence of aspergilli and penicilli on the decaying leaf litters.

Conclusion

Results of this study show that bacteria and fungi are associated with decomposition of *E. camaldulensis* and *T. grandis* leaf litters. It is clear that decomposition led to the loss in mass of leaf litters of *E. camaldulensis* and *T. grandis*. Advancement in the decomposition of the leaf litters caused a change in the microbial population and amount of CO_2 evolution, which is opined to be a consequence nutrient enrichment from the decomposed leaf litter. Since there was more decline in the mass of leaf litters of *T. grandis* than that of *E. camaldulensis*, it could be concluded that varietal microbes are essential

community of decomposition of the leaf litters of *T. grandis* and *E. camaldulensis.*

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