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Identification of predominant *Bacillus spp* under different vegetation types via 16S rRNA gene sequencing technique

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Abstract

This research was carried out in the Faculty of Agriculture Teaching and Research Farm and Forest Arboretum, University of Benin Nigeria to isolate and identify the predominant *Bacillus species* under different vegetation types using the molecular technique. Soil samples were collected from three different vegetation types at 0 - 15 cm, 15 - 30 cm, 30 - 45 cm depth. The samples were analyzed for both its physical, chemical and microbial properties. The bacteria species were identified using physiological, morphological and biochemical characteristics as well as the amplifying of the 16S rRNA through gene sequencing. The results show that microbial population, pH, TOC and other chemical properties of the soils varied under the different vegetation in response to organic matter content/crop residue, and land management practices. *Bacillus sp* and *Staphylococcus sp* were, however, the primary isolates identified from the laboratory culture while 43 bacillus isolates based on strains were identified via the 16S rRNA gene sequencing technique with the *B.cereus* (ATCC14579, JCM2152, CCM2010, NBRC1530, & IAM12605) being the most dominant among the strains.

Keywords: Bacteria strains; Vegetation; Isolate; Bacillus sp; Gene sequencing

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1.0 Introduction

Soils are highly complex, heterogeneous matrices which shelter a vast diversity of organisms (Mummey *et al.*, 2006). With thousands of different species hosted per gram of soil, microbial communities account for a large part of this biodiversity (Roesch *et al.*, 2007). As a significant component of the biosphere, soil also offers support for plant and animal development, human activities, and is directly exposed to variations in climatic conditions. It is consequently a dynamic environment, and there is a considerable body of evidence that soil microbial diversity responds strongly to changes in soil conditions (Lauber *et al.*, 2013, Tardy *et al.*, 2013).

Major interrelated factors affecting microbial diversity in soil include soil-forming processes, physical and chemical properties of soil, soil particle size distribution, vegetation and land use type. The relative effects of these factors differ in different soil types, horizons and climatic zones (Garbeva *et al.*, 2004).

The productivity and health of agricultural system are dependent upon the functional processes of the soil microbial communities, and some agricultural practices may put these communities at risk. Frequently, terrestrial ecosystem diversity is being reduced by replacing indigenous

flora with a few crops. The ecological consequences of such transitions have been addressed in several studies focusing on land degradation (Munson *et al.*, 2011, Saygin *et al.*, 2011) losses of macro biodiversity, nutrient exhaustion in soils, sustainability and restoration.

Also, soil microorganisms, including protozoa, fungi, bacteria and archaea, are essential for the proper functioning and sustainability of ecosystems (Choudhary *et al.*, 2011, Miransari, 2011).

Moreover, a high microbial diversity is assumed to be critical for the stability of ecosystems by providing functional diversity and redundancy. The influence of land use and management on soil microorganisms had been addressed in several recent studies (Peixoto *et al.*, 2010; Bissett *et al.*, 2011). However, the information acquired is still not sufficient as a systematic identification of taxa responding to the transition in land use had not been investigated.

Land-use change is one of the main factors affecting the biodiversity and functioning of terrestrial ecosystems. Soil-residing microorganisms are living parts of soil ecosystems and have complex interactions with their environment; they can directly influence soil ecological processes and maintain soil stability.

Therefore, changes in land use with different intensity or

history of agricultural practices consequently results in unprecedented changes in microbial community composition and function (Grayston *et al.*, 2004) dynamics and organic-matter storage in soils across a range of habitats (Garcia-Oliva *et al.*, 2006; Monkiedje *et al.*, 2006), which are commonly viewed as significant factors causing shifts in microbial community composition.

Knowledge of the influence of land use on the composition of the microbial population is vital for the development of a more rational method to diagnose problems, improve crop productivity and develop bioremediation strategies. It is on this note that this present experiment was carried out to examine the pattern and distribution of *Bacillus* (bacteria) species/strains under different vegetation types.

2.0 Materials and Methods

2.1 Site description

The study was carried out in the Faculty of Agriculture, University of Benin, Benin City, Nigeria. The soils of this area are predominantly acid and are mostly classified as Ultisols and Oxisols (Ogunkunle, 1993). The soils are usually fragile, very deep with a reddish colour (Ekundayo and Obuekwe, 1997). The experimental site lies between latitude 6°31m17.4N - 6°31m24.6N and Longitude 5°37m11.5sE - 5°37m13.1sE.

2.1. Sample collection

Soil samples were collected from three different vegetation types (arable cropland, a citrus orchard and forest vegetation) in the Faculty of Agriculture, University of Benin, Benin City.

The sampling was done at a soil depth of 0 - 15 cm, 15 - 30 cm and 30 - 45 cm. The soil samples were analyzed for their physical, biological and chemical properties.

2.3. Physical and Chemical Properties of the soil

The soil samples collected were air-dried at room temperature for a week; it was after that crushed to pass through a 2mm sieve. A gravimetric property of the soil was determined by the methods of Gee and Or (2002).

The organic carbon content of the soil was determined by Walkley Black (1934) wet oxidation method. The total Nitrogen was determined using the micro Kjeldahl digestion method (Bremner and Mulvaney, 1982), and the available Phosphorous was determined by the method of Bray and Kurtz (1945). Exchangeable cations (Ca^{2+} , Mg^{2+} , K^{2+} , Na^{2+} ,) were extracted with 1 N ammonium acetate solution, and the concentration of Ca^{2+} and Mg^{2+} in the soil were determined by Atomic Absorption Spectrophotometer (AAS) as described by Ramirez-Munoz (1968) while potassium (K) sodium (Na) were determined with a flame photometer (Thomas, 1982). Exchangeable acidity was determined by the KCL volumetric procedure by Mclean (1982). The soil pH was determined in 1:1 soil to water ratio using an electrode pH meter as described by Thomas (1982). Electrical Conductivity (EC) was also determined by inserting an electrical conductivity meter into the above suspension, and Exchangeable Cation Exchange (ECEC) was determined by the summation method (Maclean, 1972).

2.4. Isolation and characterization of bacteria

Microbial isolates from soil sample were done using the 'pour plate method'. The isolates were identified by standard microbial techniques as described by Cowan and Steel

(1970) by observing each isolate under a high power microscope under oil immersion (Buchanan and Gibbons, 1974) and the isolates were characterized based on cultural, morphological and biochemical differences. After that, molecular identification of the organism (*Bacillus* species) was made through gene sequencing. The genomic DNA was extracted while the primers SR (5'- AGACCCG-GAACGTATTCA) and SF (3' - GTGCCAGCACCCAC-GCTAA) were used in amplifying the 16S rRNA gene (Weiburg *et al.*, 1991). The 16S rRNA gene sequences were further analyzed using the gapped BLASTn (<http://www.ncbi.nih.gov>) search algorithm and aligned to their nearest neighbours, and the resulting organisms were selected at 99% accession.

2.5. Statistical analysis

Some Data generated were subjected to statistical (correlation) analysis using Genstat version 8 (2012), while others were analyzed using descriptive statistics.

3.0 Results and Discussion

3.1. Some Physical and Chemical Properties of Soil

Table 1 below shows some physical and chemical properties of the soil samples obtained at the different depths (0 - 15 cm, 15 - 30 cm and 30 - 40 cm) for the three vegetation types.

The pH values indicated that the soils under the study vegetation were generally acid. This is true, owing to the soil type (Ultisol), which is highly weathered and prone to leaching as recorded by Ogunkunle (1993). However, soils from the forest vegetation were less acidic, with pH ranging from 5.4 to 5.62 across the different depth, whereas the soils from the citrus orchard had pH ranging from 4.85 - 5.31. Soils from arable land appear to be more acidic in comparison with soils from other vegetation types this is very evident given that, the lowest range of pH (4.60 - 4.87) was recorded under this vegetation type and the reason for this result could be traced to high leaching and erosion of the arable land due to low vegetation cover and the effect of inorganic fertilizer frequently used in the environment for crop production. This finding agrees with the findings of Adeduntan (2010). However, the relatively less acidic pH (5.40 - 5.62) of the forest vegetation could be traced to the presence of high vegetation cover, which helps to reduce leaching and erosion.

The result for soil particle size analysis depicts that the soils from the different land use are characteristically loamy sand and no drastic change in soil texture occurred under any specific vegetation type.

Generally, a decrease in Ca and Mg content resulted in low pH (acid condition). Values for these two mineral elements were observed to change with corresponding changes in pH. As the values increased from arable land through the citrus orchard to forest land for Mg, appreciable increase for pH was noticed. Similarly, the same situation was observed for Ca. Al and H content of the soil indicates exchangeable acidity of such soils. Combined values of H and Al shows that exchangeable acidity (EA) increased as soil depth increased for Arable land and citrus orchard, whereas, EA increased from depth of 0 - 15cm to 15 - 30cm and decreased after that at a depth of 30 - 45 cm for forest land. On the other hand, ECEC had the highest value of 4.22 cmol/kg and the lowest value of 1.72 cmol/kg throughout the three vegetation types.

A similar trend of decrease in N, P and K content were

observed for the various land use as the depth increased. However, soils from forest land had a higher value of N (0.37 – 1.62 gkg⁻¹) and K (0.80 – 0.10 cmolkg⁻¹) compared to the other land use types.

The lower P values in the citrus orchard, however, could have resulted from the high plant usage of P for root and fruit development while the higher P content recorded for arable land could probably be due to the application of inorganic fertilizer over time.

Organic carbon was observed to be highest in forest land (16.00g/kg) and lowest in arable soil (0.60g/kg). However, the organic carbon value for the various vegetation decreased with increasing depth. The high organic carbon observed in the forest land could be attributed to the heavy leaf litter produced by the trees. This result is following the findings of Wang *et al.* (2017), who reported that the

carbon sources available in the soil highly depend on input from above-ground sources.

3.2. Correlation analysis between some chemical properties and soil organisms

From the correlation analysis (Table 1), there was a negative (-0.991) and highly significant (p-value<0.001) correlation between pH and CFUs of bacteria for arable land. This was also recorded by Arun *et al.* (2014). This means that the bacteria population decreased with increasing acidity (pH) and vice-versa. The high pH variability down the soil profile could be due to leaching effects (Ogbemudia *et al.*, 2016). This was observed for forest land, but the interaction was not significant. On the other hand, the negative and highly significant correlation between pH and bacterial population in the arable land could be traced to high human activities and disturbance as stu-

Table 3: Some Physical and Chemical Properties of Soil

	Depth (cm)	Sand	Silt	Clay	pH (1:1)	N	TOC	P	S	K	Mg	Ca	Na	H ⁺	Al ³⁺	ECEC
		g/kg	g/kg	g/kg		g/kg	g/kg	Mg/kg	Mg/kg	Cmol/kg	Cmol/kg	Cmol/kg	Cmol/kg	Cmol/kg	Cmol/kg	Cmol/kg
Arable Land vegetation	0 - 15	820.70	28.50	150.80	4.87	0.50	4.80	52.97	14.97	0.08	0.32	2.41	0.15	0.10	0.30	3.36
	15 – 30	751.20	25.50	223.30	4.60	0.34	3.20	34.39	19.01	0.07	0.32	1.92	0.12	0.40	0.00	2.83
	30 – 45	720.70	17.50	261.80	4.71	0.06	5.60	17.67	15.17	0.07	0.48	1.76	0.16	0.70	0.02	3.38
Forest Land vegetation	0 – 15	848.20	37.00	114.80	5.50	1.62	16.00	29.80	21.44	0.10	0.96	3.05	0.16	0.30	0.20	1.72
	15 – 30	838.20	17.00	144.80	5.40	0.37	3.80	9.65	15.37	0.90	1.16	1.92	0.15	0.30	0.60	4.22
	30 - 45	832.20	13.00	154.80	5.62	0.28	0.64	6.13	10.52	0.80	0.32	1.76	0.17	0.40	0.00	2.73
Citrus orchard vegetation	0 – 15	822.70	25.50	151.80	5.31	0.53	5.40	7.96	19.62	0.10	0.97	1.76	0.11	0.40	0.00	3.34
	15 – 30	810.00	18.50	171.80	4.97	0.27	2.60	7.39	14.36	0.07	0.97	1.12	0.13	0.60	0.00	2.89
	30 – 45	804.20	14.00	181.80	4.85	0.06	0.64	6.40	11.73	0.06	0.49	1.44	0.11	0.40	0.20	2.50

dents regularly cultivated the land for experimental purposes.

The relationship between N and the bacterial population was negative and highly significant for arable land but positive and highly significant for forest land. Nevertheless, the correlation was positive and non-significant for the citrus orchard.

A positive and highly significant relationship in P and the bacterial population was observed for both arable and forest land while the correlation was positive and non-significant for the citrus orchard.

There was a negative and non-significant relationship between K and bacteria for arable land. On the other hand, there was a negative and highly significant relationship between K and CFUs of bacteria in forest land while in the citrus orchard, the relationship was positive and non-significant. For arable land, the correlation between TOC and bacteria population was negative and highly significant. This could be as a result of other factors that affect bacteria growth such as human disturbance (Ingham, 2000). Forest land, on the other hand, had a positive and highly significant correlation between TOC and CFUs of

bacteria. This could be attributed to heavy litters produced by forest trees which decompose to release organic carbon and in turn provides a healthy environment for bacterial growth. Therefore, as the TOC increases the bacteria population also increased as other factors that affect bacterial growth are stable since the forest land usually is undisturbed. In contrast, the correlation was positive and non-significant for the citrus orchard.

Fig. 1 shows the order of the bacterial population in the various soils. The pattern of bacterial distribution in the arable land was in the following order of soil depth: 30 – 45 cm > 0 - 15 cm > 15-30 cm. The reason for the high population of bacterial at the 30 - 45 cm soil depth than the other shallow depths could be traced to the regular tilling (tillage) activities by students who regularly cultivate the land. This aligns with Muñoz-Leoz (2011), who states that conventional tillage decreases soil microbial biomass and activity. Contrarily, bacteria population was most significant at the topsoils (0 - 15 cm) for both forest land and citrus orchard, and it was observed to gradually reduced as soil depth increased this could be attributed to litter quality. Zhang *et al.*, (2013) and Zhong *et al.*, (2012) showed that litter quantity influenced soil microbial community

Table 1: Correlation analysis between some chemical parameter and soil organisms.

Correlation factors		Arable Land Bacteria	Forest Land Bacteria	Citrus Orchard Bacteria
pH	p-value	<0.001	0.3826	0.8044
	Correlation value	-0.991	-0.332	0.303
N	p-value	<0.001	<0.001	0.9368
	Correlation value	-0.929	0.995	0.099
P	p-value	0.00417	<0.001	0.926
	Correlation value	0.845	0.989	-0.116
K	p-value	0.18013	<0.001	0.797
	Correlation value	-0.490	-0.921	0.314
TOC	p-value	<0.001	<0.001	0.9112
	Correlation value	-0.922	0.996	0.139

structure and shift in the soil microbial population. While the investigation reports by Ingham (2000) suggested that an undisturbed soil often have more microbes at the top-soil than below.

3.3. Cultural vs Molecular Identification of Soil Organisms

Table 2 shows the bacteria isolates using both phenotypic and genotypic properties. Result of the DNA sequencing/identification of bacteria species shows more species rich-

ness than the usual summary presented by the biochemical and morphological characterization method of identification. This result aligns with the findings by various authors whose reports had shown that with the use of molecular methods of identification of soil organisms, over 90% of the microorganisms that can be observed microscopically (*in-situ*) could be extracted and analyzed (Steffan *et al.*, 1988; Tasi and Olsen, 1992; More *et al.*, 1994; Zhou *et al.*, 1996; Proteous *et al.*, 1997) as compared with less than 0.1% of microorganisms observed in soil that can be re-

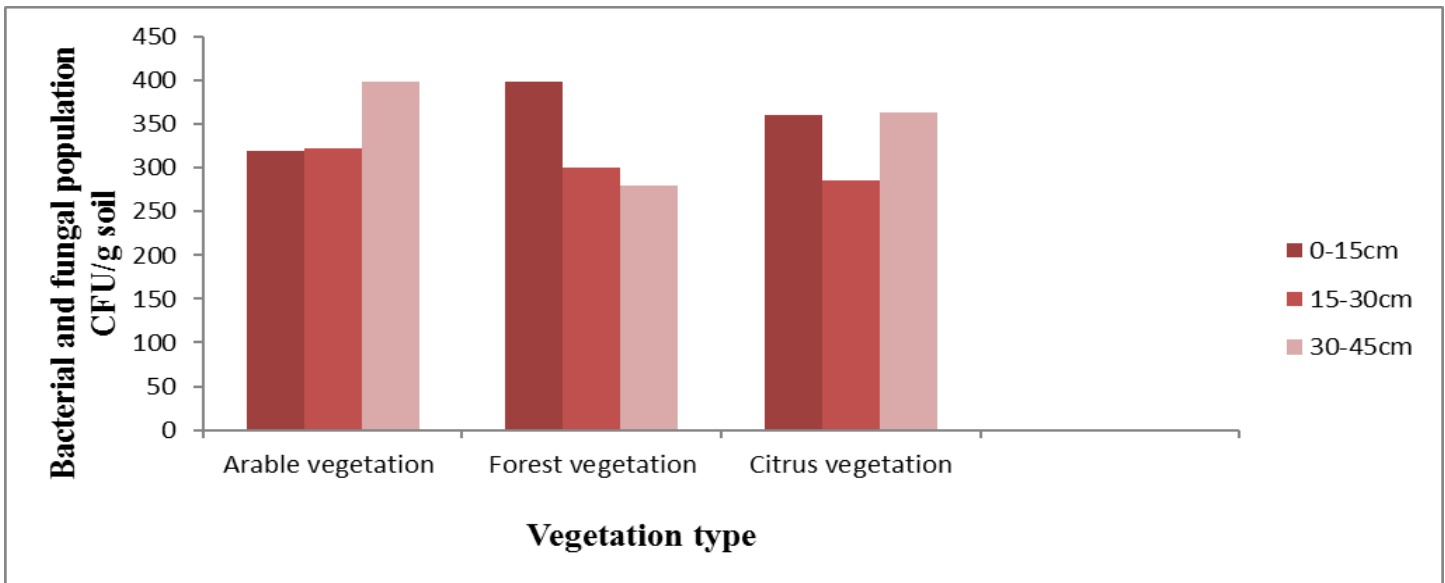


Figure 1: Bacteria population under the various land use type

covered on culture media. This report is also following the findings of Colin *et al.*, (2013) who upon the identification of bacteria communities in association with commercial salad leaf vegetables using both phenotypic and genotypic characteristic reported the cultured isolates to belong to six major bacterial phyla whereas, eleven other different phyla and subphyla were identified by culture-independent pyrosequencing. Also, Torsvik *et al.*, (1990), Atlas and Bartha, (1993.) estimated that less than 0.1% of the microorganisms found in typical agricultural soils are culturable. From the results of the DNA sequencing presented in table 3 below, it can be deduced that the phenotypic identification which involves the; morphological, physiological, cultural characteristics and biochemical properties of the organism have limitations of not being able to identify the most species/strain (s) of the bacteria when compared with the molecular technique (Don *et al.*, 2005).

4.0 Conclusion

The present study clearly shows that vegetation type significantly influenced the soil microbial population and diversity. Changes in agricultural land use type such as the conversion of natural forest to cropland have apparent effects on soil microorganisms as well as soil chemical properties. Hence, the results from this study show that a high level of heterogeneity exists in the chemical properties of soils under different vegetation types with soil from forest vegetation possessing more superior properties for microbial growth.

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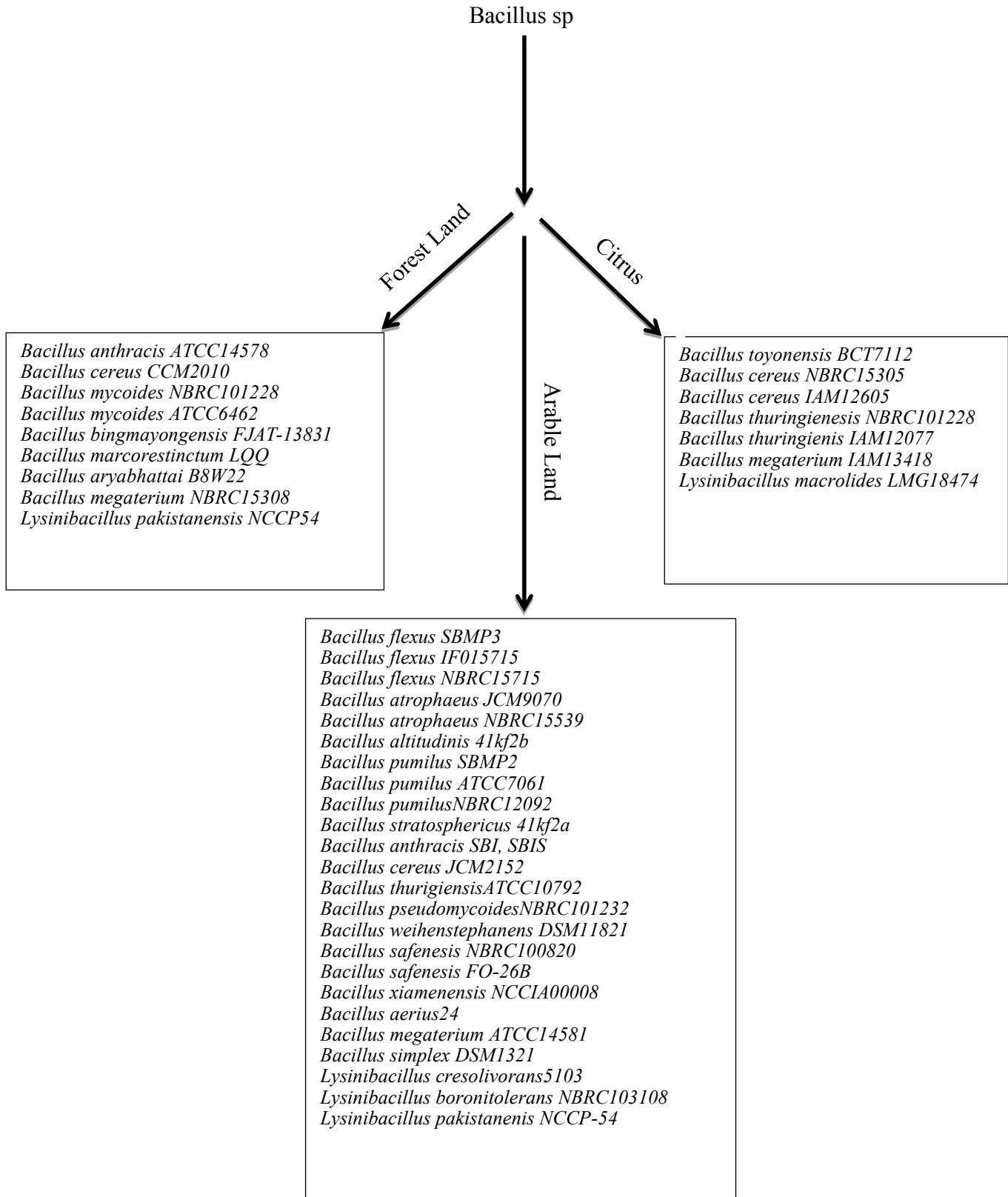


Figure 2: Genotypic isolates at 99 % identification.

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