

Assessment of Bacteria, Fungi and Protozoa in Three Theobroma Cacao Soils in Ondo State, Nigeria

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Abstract: The microbial community of 3 cocoa soils in Ondo State, Nigeria was investigated. Fourteen bacterial isolates, 8 fungi and 9 protozoa were obtained. The bacteria include *Actinomyces* sp., *Bacillus* spp, *Corynebacterium* sp., *Lactobacillus* sp., *Micrococcus* sp., *Staphylococcus* sp. and *Streptomyces* sp. The fungi were *Aspergillus flavus*, *A. niger*, *Fusarium* sp., *Penicillium* sp., *Phytophthora palmivora*, *Phytophthora* spp., *Rhizopus* sp., *Saccharomyces* sp., *Trichoderma* spp. and *Alternaria* sp., while the protozoa include *Balantiophorus*, *Biomyxa* spp, *Bodo* spp, *Colpoda* spp, *Tetramitus* spp, *Naegleria* spp and *Uroleptus* spp. Differences in population of the microorganisms in the 3 soils might be due to environmental factors of the fields, and this might account for the quantity and species of microorganisms obtained. The determination microorganism in cocoa fields is crucial as it may be exploited for the control of black pod disease, which is presently one of the most important diseases affecting cocoa production in south western Nigeria.

Keywords: Cocoa, Bacteria, Fungi, Protozoa, Ondo State, Nigeria

Introduction

Cocoa is cultivated in most tropical regions throughout the world as an economically important crop for smallholder farmers (Holmes et al., 2004). The number and kinds of microorganisms present in soils depend on many environmental factors: the amount and type of nutrients available, moisture, degree of aeration, pH, temperature among others (Prescott et al., 1999). Soil bacteria and fungi play pivotal roles in various biochemical cycles, and are responsible for the recycling of organic compounds (Wall and Virginia, 1999). The best soil for cocoa production is the forest soil rich in humus, which should be well-drained and free-flowing to allow easy penetration of roots capable of retaining moisture during summer, and those that allow circulation of air and moisture. Cocoa is grown on soils with a wide range of pH from 6.0-7.5, where major nutrients and trace elements would be available (Drenth and Guest, 2004). The beneficial roles of cocoa microbial community include organic matter decomposition, mineralization of nutrients, biological degradation and as bio-filters for cleaning

up soil and improvement of soil structure. The level of spoilage microbes reflects the microbial quality, wholesomeness of a food product, as well as the effectiveness of measures used to control or destroy such microbes (Pierson and Smoot, 2001). This work examines the microbial flora of soils from three cocoa plantations, including the protozoa which its study has received little attention. The study was done to assess the microorganisms present in these fields, in order to fully exploit their potential.

MATERIALS AND METHODS

Sample collection:

Soil samples were collected from three different fields in Odegbo, Araromi Quarters; Idele, along Supare road and a cocoa farm at Ilale, along Adekunle Ajasin University Permanent site, Akungba-Akoko, Ondo State, Nigeria. The modified methods of Mpika et al., 2011 were used for sample collection. The samples were obtained from three separate locations within a field with soil auger on topsoil at a depth of up to 10 cm, after

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removing the leaf litter to obtain random and uniform samples. In each plot, a bulk sample of 600g of soil was collected, made up of 3 samples taken at the base of 3 cacao trees bearing many healthy pods. Each bulk sample was carefully labeled, mixed and divided into three parts which were put in previously sterilized polyethylene bags.

pH readings

The Fisher Accumant pH meter (Model 600 Fisher Scientific Co, U.S.A) was used for determining the pH of the samples. Water and 0.1M KCl solution were used at 1:2.5 soil/solution ratio in a sterile beaker. The anode of the pH meter was inserted into it and the readings were obtained when it was stable.

Determination of Physico-chemical parameters

The organic carbon (OC) content was determined by the modified $K_2Cr_2O_7$ digestion of Walkley-Black wet oxidation method. Flame photometer was used for measuring Na and K, while Atomic Absorption Spectrophotometer was used for Mg and Ca.

Cultivation of microorganisms

Preparation of media for isolation

The medium used for isolation of bacteria and protozoa was Nutrient agar (NA), while Sabouraud Dextrose Agar (SDA) was used for fungi. The media were prepared according to the manufacturer's instruction and specifications, sterilized in the autoclave for 15 min at 121°C temperature and 103.42kPa pressure before allowed to cool.

Cultural methods for bacteria and fungi

The pour plate technique was used for inoculation of soil samples. Petri dishes were arranged on a working bench for each of the samples collected. One gramme of each soil sample was suspended in 9 ml of sterile distilled water, mixed thoroughly and diluted to 10^{-4} , 10^{-5} and 10^{-6} for bacteria and protozoa, while 10^{-3} , 10^{-4} and 10^{-5} were used for fungi. A 1ml aliquot was then dispensed into each sterile Petri dish and a molten NA, SDA was poured into each dish. The plates were then gently swirled for 10 s to aid even distribution of both the sample and the medium, and were allowed to cool and set before incubation. Incubation of the plates was done at 37°C for bacteria, and 25°C for fungi in an inverted position for 48 hours until reasonable growth occurred. The isolates were preserved and maintained on NA and SDA slants, and kept at 4°C

in the refrigerator until further analysis. The cultural characteristics of the colonies were observed.

Isolation and identification of protozoa

Methods of Subba Rao, 1999 was used for isolating the protozoa. *Escherichia coli*, a good example of edible bacteria for soil protozoans was used. Cultures of *E. coli* were first cultivated on NA in 9 sterile plates at 37°C for 24 hours. After the incubation, 1ml of each of 10^{-4} , 10^{-5} and 10^{-7} soil samples was transferred into each of the bacterial cultures, and appropriately labeled. The plates were sealed with masking tape and incubated for 10 days at 30°C. Staining was done by preparing a smear on microscope slides, flooded with Giemsa stain, and allowed to dry for 30 s before viewing under the microscope with oil-immersion lens. They were identified and classified into groups based on the morphology- shape and organ of locomotion: Ciliates, Amoebae (move by means of a temporary foot or "pseudopod- testate amoebae (makes a shell-like covering), naked amoebae and Flagellates (use a few whip-like flagella to move (Minchin, 2003).

Identification of bacteria

Gram's staining was done to assess the organisms that retained the purple colour of crystal violet which were considered Gram positive, and those that retained the red colour of safranine: Gram negative. Biochemical tests carried out include catalase test, sugar tests include glucose, sucrose, lactose and mannitol. Others were indole test, starch hydrolysis, sugar fermentation, motility and Ornithine tests (Ederer and Clark, 1970).

Identification of fungi

Pure cultures of fungal isolates were characterized between 48 and 96 h after incubation. They were viewed under the microscope with Lactophenol-in cotton blue, and classified based on colony types and morphology of the spores according to the descriptions of various identification books including Barnett and Hunter (1998), Williams-Woodward (2001), Dayan (2004) and Chaturvedi and Ren (2011). Radial growth of the isolates were measured daily in some cases to ease identification.

RESULTS

All the soils were observed to be acidic (pH 6.30-6.45), and more ideal for cocoa plantation. However, the acidity was higher in Odegbo, followed by Ilale and Idele. The Organic Content (OC), K, Ca and Mg

were highest in Odegbo than in other locations (Table 1).

Eight bacterial isolates, 8 fungi and 9 protozoa were obtained. The total bacterial count for Odegbo ranged from 2.0 to 4.0 x 10⁶ cfu/g, Idele ranged from 1.0 to 9.0 x 10⁶ cfu/g and Ilale ranged from 2.0 to 4.0x 10⁶ cfu/g. The highest and lowest bacterial

count, 9.0 x 10⁶ and 1.0 x 10⁶ cfu/g respectively, was observed in Idele as shown in Table 2. Odegbo and Ilale had the highest number of bacteria (6) present in the soil samples, while Idele had the least (5). *Actinomyces* sp., *Bacillus*, *Corynebacterium* sp. and *Micrococcus* sp. were obtained from all the three cocoa fields. This is an indication that these microorganisms are predominant in cocoa soil.

Table 1. Physico-chemical properties of cocoa soils

Properties	Odegbo	Idele	Ilale
Description	Beside the stream	With Palm trees	With Banana trees
pH	6.30	6.45	6.35
OC (%)	3.69	2.97	2.73
Na (meq/100g)	0.18	0.17	0.22
K (meq/100g)	0.24	0.23	0.22
Ca (meq/100g)	1.8	1.5	1.6
Mg (meq/100g)	1.3	1.2	1.2

Table 2. Population and occurrence of bacteria in cocoa soils

	Odegbo	Idele	Ilale
Total bacterial count (cfu/g)	2.0 - 4.0 x 10 ⁶	1.0 - 9.0 x 10 ⁶	2.0- 4.0 x10 ⁶
<i>Actinomyces</i> sp.	+	+	+
<i>Bacillus</i> sp.	+	+	+
<i>Corynebacterium</i> sp.	+	+	+
<i>Lactobacillus</i> sp.	-	+	-
<i>Micrococcus</i> sp.	+	+	+
<i>Staphylococcus</i> spp.	+	-	-
<i>Streptococcus</i> sp.	-	-	+
<i>Streptomyces</i> sp.	+	-	+

Keys

+ = Present, - = Absent

	Odegbo	Idele	Ilale
Total fungal count (cfu/g)	1.6 - 3.2 x 10 ⁵	1.4 - 3.0 x 10 ⁵	1.8- 3.3 x10 ⁵
<i>Aspergillus flavus</i>	+	+	+
<i>Aspergillus niger</i> .	-	+	-
<i>Fusarium</i> sp.	+	+	+
<i>Penicillium</i> sp.	+	+	+
<i>Phytophthora</i> sp.	+	-	-
<i>Rhizopus</i> sp.	+	+	+
<i>Saccharomyces</i> sp.	+	+	+
<i>Trichoderma</i> spp.	+	+	+
<i>Alternaria</i> sp.	+	-	-

Table 3: Population and occurrence of fungi in soil samples

Keys

+ = Present, - = Absent

Table 4. Occurrence of protozoa in soil samples

Protozoa	Location		
	Odegbo Stream* ¹	Idele Palm trees* ⁵	Ilale Banana* ⁴
<i>Acanthamoeba</i> spp	-	+++	++
<i>Balantiophorus</i>	+	-	-
<i>Biomyxa</i> spp.	+	+	-
<i>Bodo</i> spp.	++	+	++
<i>Cercobodo</i> spp.	+	-	-
<i>Colpoda</i> spp.	+	-	+
<i>Tetramitus</i> spp.	+	+	+
<i>Naegleria</i> spp.	-	-	+
<i>Uroleptus</i> spp.	++	+	+
<i>Euglypha</i> spp.	-	+	-

*Number of cysts formed in 3 samples, + or – Presence or absence of protozoan isolate in samples

Thirty one (31) fungi were isolated from the three fields. Odegbo had the highest number of fungi (8) present in the soil samples, while Ilale had the least (6). The highest and lowest fungal counts, 3.3×10^5 and 1.4×10^5 cfu/g were observed in Ilale and Idele respectively. *Phytophthora* sp. and *Alternaria* sp. were found only in Odegbo. *Aspergillus flavus*, *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., *Saccharomyces* sp., *Trichoderma* spp. were obtained from all the three cocoa fields, while *Aspergillus flavus*, *Rhizopus* species and *Penicillium* had the highest occurrence in all the cocoa fields (Table 3).

The study showed that flagellated protozoans (Class Mastigophora) were more predominant in cocoa soils. These include *Acanthamoeba* spp., *Bodo* spp., *Cercobodo* spp., *Biomyxa* spp., *Uroleptus* spp., *Tetramitus* spp., and *Balantiophorus* spp. (Table 4). The highest number of protozoa (7) present in the soil samples was observed in Odegbo, while Idele and Ilale had the least (6). Highest number of cysts (5) were observed in Idele, whereas Odegbo had the least (1).

DISCUSSION

The results showed a high proliferation of bacteria, fungi and protozoa with a greater proportion of bacteria. The cocoa soils are therefore one of the preferred sites of indigenous microorganisms. Most of the bacteria reported in this study have been shown to be present in cocoa soils by previous workers (Amir and Pineau, 1998). Odegbo and Ilale had higher bacteria and fungi probably due to the nature of the soil and the use of synthetic chemicals and pesticides which could adversely affect the soil microbial balance, causing the soil microorganisms to grow when they are used as carbon and energy source (Deacon, 2005). The number of microorganisms may increase depending on the organic matter content of any particular soil. The bulk of soil bacteria are heterotrophic and utilize readily available source of organic energy from sugars, starch, cellulose and protein. *Actinomycetes*, which were found in all the fields, grow on complex substances such as keratin, chitin and other complex polysaccharides, and thus play an active role in humus formation.

Soil fungi are mostly heterotrophs. Sporulating fungi such as *Mucor*, *Penicillium* and *Aspergillus* appear on agar plates rather profusely than non-sporulating ones (Saritha and Sreeramulu, 2013). According to Adebola and Amadi (2010a), *Rhizopus*

spp. could possibly serve as a good biological control agent against *Phytophthora palmivora*. Many beneficial fungi and bacteria that occur naturally and associated with cocoa had been reported to show potential as antagonists of major cocoa pathogens (Bong *et al.*, 2000; Shari Fuddin, 2000; Samuel and Habber, 2003; Adebola and Amadi, 2010b).

The highest population of protozoans found in cocoa soil belongs to flagellates (class Mastigophora). This could be due to litter content, soil depth, pore-size and water potential (Stout and Heal, 1967). Other reports highlighted that protozoan abundance and diversity may be greater in environment with relatively high level of environmental stress.

Encystment of protozoa was observed, this indicates that the cells have accumulated sufficient reserves when the conditions became unsuitable for their activities. Conversely, where nutritional resources are low, encystment is limited and many cells die if the soil dries out (Couteaux and Ogden, 1988).

CONCLUSION

Natural populations of microorganisms in cocoa soils - bacteria, fungi and protozoa were obtained in this study. Almost all the soil living organisms have different micro-environment in which they live (Rana, 2005, Subba Rao, 1999). It was observed that the total bacterial counts were higher than the fungal counts in samples from the three fields. This predominance of bacteria over fungi in cocoa soils had been observed by several authors (Okoh *et al.*, 1999). The biodiversity was variable qualitatively and quantitatively. Differences in population of microorganisms in the 3 soils might be due to physiological features of the fields, and this might account for the quantity and species of microorganisms obtained. Protozoan isolates from the field beside the stream were higher in number than for other fields. This might be due to high level of water potential which enhances movement of the organisms.

The study was done within the limits of the facilities available. Modern technology (nucleic acid probes) approach should be employed to obtain detailed overview of the microbial diversity. The potential of the isolated microorganisms, especially fungi and bacteria could be exploited in the control of black pod disease caused by *Phytophthora palmivora* and *P. megakarya*, and this will instill hope in cocoa

farmers whose revenues constantly decline due to this disease.

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