

Screening of Fungal (*Cunninghamella Bertholletiae*) Pathogenic Activity on Microbial Community in Cassava (*Manihot Esculenta* Crantz)-Cultivated Soil

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Abstract—The pathogenic activity of a fungus (*Cunninghamella bertholletiae*) isolated from cassava (*Manihot esculenta* crantz) on some soil bacterial community was determined. To isolate bacteria and fungi, 1 mL suspension of cassava cultivated soil samples (0.2 g in 250 mL) was spread-plated onto a nutrient agar containing cycloheximide (1.75mL/250mL) (for bacterial isolation) and Potato dextrose Agar (PDA) containing Ampicillin (500µL/250mL) (for isolation of fungi). The plates incubation were at 37 °C for 24 h for bacterial and 29°C for 72 h for fungal species. Pure colonies of microorganisms isolated were identified using the VITEK 2 biochemical test, morphological, structural and microscopic characteristics for bacteria and lactophenol cotton blue staining method for fungi. Results revealed bacterial communities i.e.; *Oligella ureolytica*, *Acinetobacter* family, *Sphingomonas paucimobilis*, *Myroides* sp., *Achromobacter denitrifican*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, *Sphingomonas paucimobilis*, *Methylobacterium* sp., *Pseudomonas luteola*, yeast; *Candida lipolytica* and fungi such as; *Cryptococcus albidus*, *Rhodotorula* sp.. Nucleotide sequencing and phylogenetic analysis and screening of its pathogenic activity on bacterial communities revealed that *Cunninghamella bertholletiae* (*C. bertholletiae*) is pathogenic thus, inhibits and suppresses the bacteria; *O. ureolytica*, *Acinetobacter* sp., *P. luteola*, *S. paucimobilis* and it was slightly antagonistic against *Myroides* sp., *S. maltophilia* as well as *C. lipolytica*. However, the fungus demonstrated insignificant pathogenicity against *C. albidus* and *Rhodotorula* sp. From the result of this study, *C. bertholletiae* was pathogenic against some bacteria and yeast found on cassava cultivated soils and therefore, has the potential to be used as bio-control agent for plant pathogens in agriculture, biotechnology and environmental engineering.

Index Terms— Agricultural Soil, Cassava (*Manihot Esculenta* Crantz), Fungal Pathogenic Activity, Microbial Community.

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I. INTRODUCTION

Cassava (*Manihot esculenta* crantz) is a crop from a family of *Euphorbiaceae* which constitutes a main staple food for many communities worldwide. Thus, countries from the African, Asian and Latin American continents massively produce this cultivar for self-consumption and poverty alleviation [1] as well as for the industrial production of biofuel and biogas [2], [3]. However, the crop contains cyanogenic glycosides compounds which enzymatic hydrolysis transform into free cyanide during plant's growth, accidents or external attack [4], [5]. The CN⁻ produced from the plant reaches the soil where it is accumulated and impacts negatively on the soil's microbial diversity.

Microorganisms (microbial and fungal communities) have played an important role in the environment and the sustainability of life on earth for millions of years. Bacteria may be aerobic or anaerobic, while, some are photosynthetically active, and play a role of producers of organism's food chain. Fungi are abundant in terrestrial ecosystems, and thereby often play the role of decomposers of organic and inorganic material [6]. Which in turn are transformed into plants and animals growth support habitat or components such as soil and nutrients. These materials are thereby transformed into a growth support system for plants or animals as the decomposed material, now known as humus or compost, contains essential nutrients for growth. Therefore, bacterial and fungal communities within soil influence the good functioning of the environment in general as well as the terrestrial ecosystem in particular, contributing to plant growth and productivity in the process.

The presence of microbial and fungal communities within a particular terrestrial ecosystem however, can be problematic due to the fact that certain fungi have a more rapid growth on a particular environment and substrates than other microorganisms [7], [8]. A faster fungal growth can be competitive, inhibiting and pathogenic to some bacterial species [9], [10], [11]. These pathogenic microorganisms often produce metabolic properties, antimicrobial substances (proteins/toxins), which suppress other microorganisms (i.e. bacterial yeast) growth. The presence of pathogenic fungi such as *Cunninghamella* sp. can contribute to an imbalance between the two microbial communities within cassava cultivated soils.

This paper focuses on a screening of fungal (*C. bertholletiae*), pathogenic activity on some microbial community in cassava cultivated soil. Soil microbial diversity was isolated by plating onto NA and PDA growth media followed by biochemical testing, morphological and

structural identification in conjunction with blue staining of isolates.

II. MATERIALS AND METHODS

A. Cassava plants growth

Cassava plants were cultivated in a greenhouse at the Cape Peninsula University of Technology (CPUT) where cassava cuttings, approximately 10cm long, were placed in plastic trays with a soil mix (nursery matrices). An automated irrigation system was used in order to irrigate plants three times a day. A heating fan was used to regulate the air circulation with a temperature ranging between 18-19°C at night and between 23-25°C during the day. After three to four weeks, plants were transferred to another area in the glasshouse in order to increase their exposure to solar radiation. The irrigation of plants were also reduced to twice daily a rate of 2-3 minutes per irrigation sequence, with the humidity matured at 40-60%.

After three and half months cassava plants were then transferred in small (4kg) plastic bags and placed into another glasshouse where they were watered twice a week with an organic fertilizer; 3:2:1 NPK (nitrogen:phosphorous:potassium) being added into soil as per the horticulture standards, in order to facilitate plant growth. After a five-month growth period, plants were transferred into 10kg bags in order to facilitate rooting.



Plate 1: Cassava cuttings in plastic trays



Plate 2: cassava plants in small (4kg) plastic bags



Plate 3: cassava plants in small (10kg) plastic bags

B. Analytical methods

1. Soil sample collection and preparation

Soil sample collection was done using a sterile stainless steel teaspoon at the depth of 3-5cm from the surface and placed into sealed pedlar plastic bags. The plastic pedlar bags were then placed into an empty cooler box to avoid any solar radiation and taken to the laboratory where samples were refrigerated at 4°C prior to further analysis. A small pinch (0.2g) of each soil sample (silt) was poured into a clean 250 mL sterile Schott bottle containing deionised distilled water in order to obtain a 10×10^3 dilution. A clean 250 mL sterile Schott bottle of Nutrient Agar was used to dilute

cycloheximide (1.75mL/250mL) while, Ampicillin (500μL/250mL was diluted into Potato dextrose Agar (PDA) after autoclaving at 121°C for 15 min. The warm contents of the NA and PDA were then poured into clean petri dishes, respectively, and the eluent (10×10^3 dilution) spread-plated onto the respective using a sterile glass spreader. Plates were incubated at 29°C using an ITF 80L incubator (PROLAB instruments: Model 226/296) for 72h depending on bacterial growth.

2. Microbial Isolation and Identification

After sufficient growth were observed (approximately 72hours), microorganisms were sub-cultured several times to obtain pure isolates. Pure colonies of microorganisms (bacteria and fungi) were then identified and characterised on basis of their morphological characteristics and microscopic structure in terms of either conidial shape (fungi) and colour (gram + or gram-) (bacteria) observation using an Olympus CX21-model CX21FS1 microscope (Olympus corp., Tokyo- Japan) ; using staining method. Fungal identification was done by staining the pure fungal isolates (Figure 1) with a lactophenol cotton blue staining reagent on a glass slide. Further biochemical identification for both bacterial and fungal communities was done using VITEK 2 Systems Version: 07.01 (CPUT AgriFood Technology Laboratory).

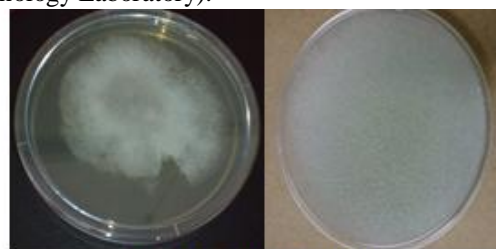


Fig. 1 *C. bertholletiae*



C. bertholletiae top view



C. bertholletiae bottom view

3. Pathogenicity screening of *C. bertholletiae* on bacterial and yeast communities

Fungal mycelia (0.2-0.5mg) was placed at the centre of fresh PDA growth media plates containing different bacterial isolates, respectively, and labelled accordingly. The plates were incubated at 29°C as optimum growth temperature for growing fungi and bacteria at the same time and incubated in an ITF 80L incubator (PROLAB instruments: Model 226/296) for 168h depending on bacterial growth in order to assess fungal antimicrobial activity at 24h, 48h, 120h and 168h intervals (Figures 2 and Appendixes).

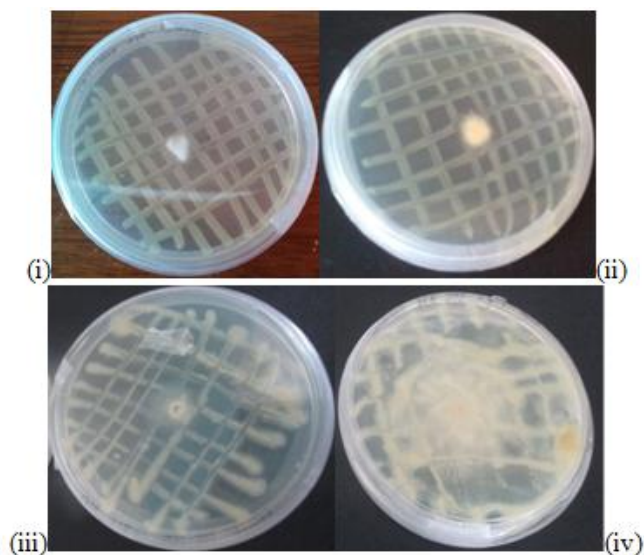


Fig. 2a Cultivation of *Oligella ureolytica* after (i) 24h, (ii) 48h, (iii) 120h and (iv) 168h

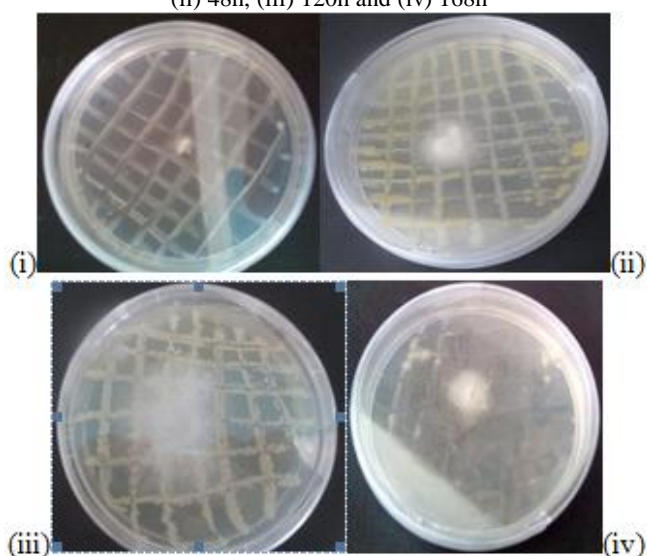


Fig. 2b Cultivation of *Acinetobacter* sp. after (i) 24h, (ii) 48h, (iii) 120h and (iv) 168h

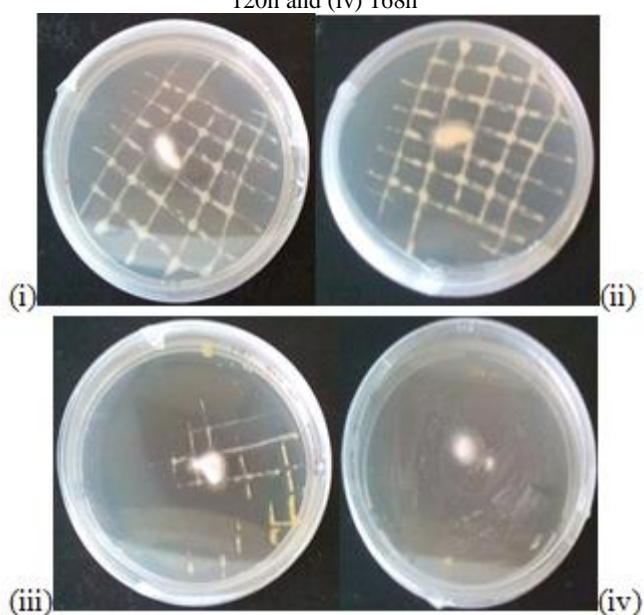


Fig. 2c Cultivation of *Pseudomonas luteola* after (i) 24h, (ii) 48h, (iii) 120h and (iv) 168h

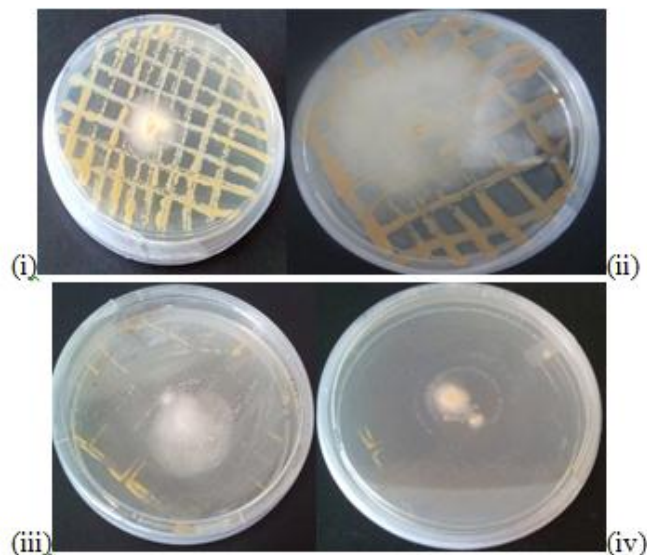


Fig. 2d Cultivation of *Sphingomonas paucimobilis* after (i) 24h, (ii) 48h, (iii) 120h and (iv) 168h

4. Preparation of bacterial and fungal samples for biochemical analysis using VITEK 2 systems

VITEK 2 is (BioMérieux, Inc – France) an automated system used for accurate microbial phenotype identification. The instrument is equipped with advanced software and uses colorimetric reagent cards [13] for the specific identification process of several microorganisms i.e. bacteria, fungi and yeasts. Bacterial species were cultured on Nutrient Agar containing cycloheximide (1.75mL/250mL) while, fungi and yeasts were placed onto Potato dextrose Agar (PDA) with Ampicillin (500μL/250mL) and incubated between 24h to 48h at 29°C in an ITF 80L incubator (PROLAB instruments: Model 226/296) depending on microorganisms growth. Thus, microorganisms were sub-cultured several times in order to get pure colonies. Pure bacterial and yeast cultures of 16h growth were taken to the CPUT AgriFood Technology Laboratory for further biochemical analysis (using VITEK 2 Systems Versions 07.01).

C. Kinetic study of biological process

Biological process (bioprocess) is important due to its role in the sustainability of life in general and for the prediction as well as control of processes that influence life in particular. It is often used in various studies under controlled environment for accuracy purposes. Thus, kinetic study of bioprocesses can be categorised as structured and non-structured models. The latest is often used and considered easy for manipulation [12]. Therefore, a non-structured model (such as Monod's model) is mostly used in microorganisms' kinetic growth [14], [15]. The mathematical formulas (equations) below are often used to summarise the non-structured method.

$$\mu = \mu_m (S / K_s + S) \quad (\text{Equation 1})$$

With K_s being the constant applicable while,

$$\mu = \frac{1}{2} \mu_m \quad (\text{Equation 2})$$

The above is the description of a microorganism's growth rate which is susceptible to decrease in relation to growth media (or competitive organisms) is depleted and vice versa.

III. RESULTS AND DISCUSSION

A. *C. bertholletiae* pathogenic ability on bacteria and yeast

Cunninghamella bertholletiae's pathogenic ability on bacterial diversity (*Oligella ureolytica*, *Acinetobacter* sp., *Pseudomonas luteola*, *Sphingomonas paucimobilis*, *Myroides* sp., *Achromobacter denitrificans*/*Achromobacter xylosoxidans*, *Methylobacterium* sp., *Stenotrophomonas maltophilia*), yeasts such as *Candida lipolytica* and fungi such as *Rhodotorula* sp. and *Cryptococcus albidus*, was tested for the duration of 24h to 168h. The results revealed that *C. bertholletiae* was pathogenically active on some bacterial community but, it was slightly aggressive against *Myroides* sp. (Appendix A), *S. maltophilia* (Appendix D) as well as *C. lipolytica* (Appendix E). While, it was less fungicidal on the *Rhodotorula* species and *Cryptococcus albidus* was insignificant.

Cunninghamella bertholletiae's pathogenicity on *Pseudomonas luteola* and *Sphingomonas paucimobilis* was imminent and noticeable at 24h after the inoculation (Figure 2c,d). At 48h the fungus showed some growth and antimicrobial activity as bacterial sizes decreased. At 120h, fungal growth started to slow as bacterial content in the media decreases to become totally stagnant and even smaller at 168h as bacterial growth was completely depleted (Figure 2c,d). Fungal growth was observed after 48h with significant antimicrobial activity on *Acinetobacter* sp. (Figure 2b), but not noticeable with *Oligella ureolytica* (Figure 2a). After 120h, pathogenic activity were however observed with both bacterial organisms even though the *Acinetobacter* sp. was more susceptible (or sensitive) than *Oligella ureolytica*. After an incubation period of 168hrs, fungal growth in the presence of the *Acinetobacter* sp. was barely visible (Figure 2b), while *Oligella ureolytica* could still be observed (Figure 2a).

Cunninghamella bertholletiae displayed significant growth and antimicrobial effect on the microbial species that were exposed to the fungus i.e. the *Myroides* sp., *Achromobacter denitrificans*/*Achromobacter xylosoxidans*, *Methylobacterium* sp., *Stenotrophomonas maltophilia* and *Candida lipolytica* after 24h of inoculation (Figure 2b,c,d; Appendix A). After 48h and 120h exposure times, respectively, the fungus' pathogenicity became significantly evident on the *Stenotrophomonas maltophilia* and *Candida lipolytica* growth plates, which was characteristic of the diminished microbial growth patterns of the respective microorganisms also by their physical destruction (disappearance).

Achromobacter denitrificans/*Achromobacter xylosoxidans* displayed different characteristics which resembled or could be regarded as antagonistic/bacterial resistance behaviour. This was concluded as a result of fungal growth ceasing and significant changes in bacterial colour where a bacteria-fungus connection occurred (Appendix B). However, both *C. bertholletiae* and *Methylobacterium* sp. displayed a similar degree of growth which was not affected by the presence of either species as compared to the other abovementioned microbial species. Instead there is a kind of symbiosis [16], [17] – mutual and beneficial cohabitation between a fungus (*C. bertholletiae*)

and bacteria which is characterised by mutual growth of both species (Appendix C) [18], [19].

However, there were a variation in shapes amongst the microorganisms' from i.e. rods (*Oligella Ureolytica*, *Candida lipolytica*; cocci (*Acinetobacter* family); rods, chains some singular (*Achromobacte* sp.); to rods, small, chains and singular (*Methylobacterium* sp.) and long rods (*Rhodotorula* species and *Cryptococcus albidus*).

Thus, *C. Bertholletiae* pathogenic activity within agricultural soil has negative effects on soils bacterial community; - it suppresses bacterial growth of i.e. *Pseudomonas luteola*, *Acinetobacter* sp, and *Sphingomonas paucimobilis* species (Figure 2b,c,d). However, it is known that the absence of some bacteria community in soil can negatively affect soil quality and plants growth as some endophytic microorganisms contribute in plants biocontrol/bio protective mechanism- prevent plants diseases [20], [17]. Thus, a decrease in microorganism's community will contribute not only in lowering soils biological (biomass, organic matter formation and content) and physical properties such as; soil aggregate formation, aeration, compaction, water circulation abilities [21] but, as well in increasing plants vulnerability to diseases and external attack-therefore, lead to low crop production and yield.

Whereas, previous studies on bacterial/fungal interactions (BFI) have demonstrated that it is important for the environment, in agriculture and in human health [21], [17]. Thus, the interaction was used as a basis in promoting new and advanced technology and engineering studies such as; discovery and identification of new antibiotics used in humans therapeutic as well as in agriculture field. In agricultural and environmental engineering, BFI are used in improving mushroom production, plants and animal diseases [22], [23], [16]. The fungus can be used as bio-control agent for plant pathogens in prevention and treatment. On cassava tubers its can be used as post-harvest prevention/treatment against bacterial attack - thus, improve and increase products quality and productivity.

B. Biochemical analysis using VITEK 2 Systems Versions 07.01

An identification card was used for the identification of all bacterial species while, a yeast identification card was used for yeast/fungal identification. Results showed excellent identification with 95-99% probability for all bacteria and 86% acceptable probability for yeast/fungi. The Biochemical identification (Biochem ID) system revealed that all microorganisms (bacteria and yeast) were Gram- similar identification to the microscopic ID except the two fungi (*Rhodotorula* species and *Cryptococcus albidus*) that were Gram+.

IV. CONCLUSION

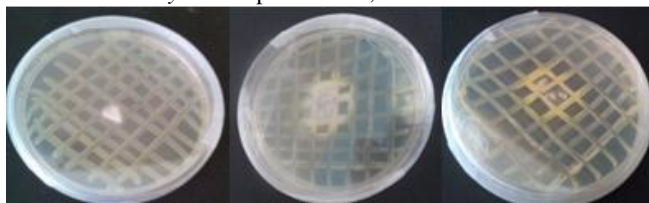
A fungus *C. Bertholletiae* demonstrated significant pathogenic activity on bacterial species such as; *Oligella ureolytica*, *Acinetobacter* family, *Pseudomonas luteola*, *Sphingomonas paucimobilis*, *Myroides* sp., *Stenotrophomonas maltophilia* and yeast i.e.: *Candida lipolytica*. Although, it showed less growth and antimicrobial activity on bacteria such; *Achromobacter denitrificans*/*Achromobacter xylosoxidans* while, its

interaction with *Methylobacterium* sp. revealed a mutual and beneficial symbiosis. Whereas, it's pathogenic activity on soil microbial community does not only lead to suppress their growth but, also results in a total destruction of microorganisms. Thus, contributes to a decrease of microorganisms within a cassava cultivated soil. A decrease in microorganism's community will contribute not only in lowering soil's bio-physical properties such as; organic matter formation, biomass and OM content, aeration, compaction, water circulation abilities [21] but, also increase plants vulnerability to diseases and external attack-therefore, lead to low crop production and yield. While, the interaction *C. Bertholletiae* and soil organisms (bacteria) can be used in order to enhance advanced environmental and agricultural engineering.

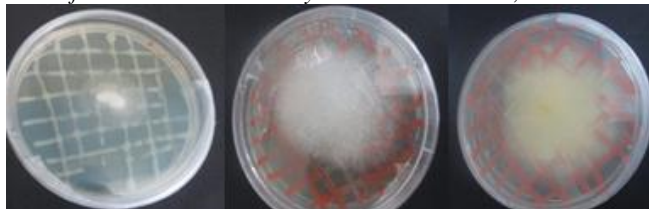
APPENDIXES



Appendix A *C. bertholletiae* pathogenic activity on *Myroides* sp. after 24h, 48h and 120h



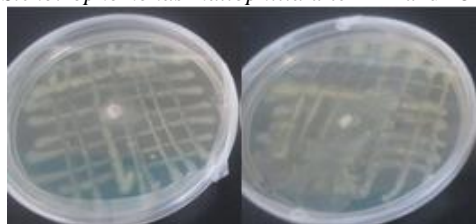
Appendix B *C. bertholletiae* pathogenic activity on *Achromobacter denitrificans/Achromobacter xylosoxidans* after 24h, 48h and 120h



Appendix C *C. bertholletiae* pathogenic activity on *Methylobacterium* sp after 24h, 48h and 120h



Appendix D *C. bertholletiae* pathogenic activity on *Stenotrophomonas maltophilia* after 24h and 48h



Appendix E *C. bertholletiae* pathogenic activity on *Candida lipolytica* after 24h and 48h

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