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**Research Paper**

# Identification and morphological characterisation of bacteria causing cassava root necrosis disease in Tshopo province, Democratic Republic of Congo

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***Summary***
*The purpose of this study is to identify the bacteria that cause cassava root necrosis disease (CRND) in the Province of Tshopo in the Democratic Republic of Congo. To accomplish this, a survey was conducted in three cassava collection fields: WAVE/IFA- Yangambi, IITA, and INERA-Yangambi. 30 cassava tubers with necrosis signs were collected from each collection field and transported to the Plant Phytopathology and Biotechnology Laboratory of WAVE/IFA- Yangambi to be cultured on the LPGA culture medium and examined macroscopically and microscopically. The severity of cassava root necrosis disease was determined for the three collection fields and is provided as follows: The WAVE/IFA- Yangambi collection field has a severity of 3, whereas the IRERA- Yangambi collection field has a severity of 2, and IITA has a severity of 2. The disease was severe in the WAVA/IFA-Yangambi collection field, but less so in the other two. Macroscopic and microscopic analyses of isolates from necrotic cassava roots in the laboratory showed the species of bacteria responsible for this disease. These are bacteria belonging to the genus Xanthomonas.*

***Keywords:*** *Bacteria, Root necrosis, Cassava, Collection fields, Tshopo*

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## I. Introduction

Cassava is an important food for about 800 million people in tropical regions, including 500 million in Africa (Vernier & Zakhiarosis, 2018). In 2019, DR Congo was rated second in Africa, producing 40 million tonnes yearly, after Nigeria (59 million tonnes) (FAOSTAT, 2021). Cassava is an essential source of metabolizable energy due to the high starch content of its tuberous roots (FAO, 2013). Tuberous roots additionally contain significant quantities of vitamins C, thiamine, riboflavin, and niacin. According to Tshingombe et *al*. (2008), cassava is the most often eaten energy food in the DRC, at 74.4%, followed by corn at 68.8%. Cassava usage is steadily growing (2.5 to 3% each year), because ofanincreasein the population depending on cassava for food (Cock, 1985). According to the FAO (2000), cassava provides an income for almost 70% of the Congolese people. Cassava is becoming increasingly popular among farmers, mostly for commercial or subsistence uses (PRONAM, 1984; Berry, 1993). Cassava production in Central and Eastern Africa is severely impacted by two viral diseases: cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). These two viruses generate one billion US dollars in yearly losses (IITA, 2014) and threaten food security in sub-Saharan Africa (Patil et *al*., 2015).

Cassava root necrosis disease (CRND) is widespread in the Democratic Republic of Congo. At the beginning of the 2000s, a disease related to cassava brown streak (named CBSD-like) was identified in cassava

fields in Kinshasa and Kongo-Central (Mahungu et *al*., 2003). This disease, however, has not established any stem or foliar symptoms, unlike CBSD, which does in East Africa (Bakelana, 2022). Due to a lack of molecular analysis and following the similarity of root symptoms with CBSD, the existence of a virus has always been suspected to be the cause of the spread of this CBSD-like disease(Bakelana et *al.*, 2020).

Using CBSD virus-specific primers, PCR diagnostics failed to detect or identify viral infections in symptomatic cassava samples obtained in western DRC (Bakelana et *al*., 2019b). As a result, next-generation sequencing (NGS) techniques have been developed and implemented, as they can sequence complete species' genomes. These methods are commonly used to identify microorganisms causing new diseases (Bakelana, 2022).

Several attempts to identify the agent responsible for CRND in the western DRC have failed (Bakelana et *al*., 2019b). Molecular diagnostic findings from five independent laboratories employing PCR primers specific for two known CBSVs (CBSV and UCBSV) came back negative for CBSVs. This implies that the causal agent of CRND may differ from those currently identified (Bakelana et *al*., 2019c).

Given the futility of viral detection, we suspect the necrosis observed on cassava roots is fungal and/or bacterial in origin.

This study focuses on this new disease that is causing the loss of cassava production. For this purpose, cassava collection fields were investigated to identify the microorganisms causing cassava root necrosis disease (CRND).

## II. Materials and methods

### 2.1. Prospection

Data on the occurrence of root necrosis disease were collected in three fields. The first is that of WAVE located at Pk14 on the old Buta road axis (Latitude N 0°36.01' 872"; Longitude E 25°15'53.83656"; Altitude: 400.8m), the second is that of the IITA located at Pk43 of Kisangani on the Ituri road axis (Latitude N 0°28'58"; Longitude E 25°31'638"; Altitude: 482m; precision: 4.94m), and the third is located in the city of Yangambi, approximately 100 km northwest of the city of Kisangani, on the right bank downstream. This collection was done from March to May 2023.

### 2.2. Material

In this study, tubers from various cassava cultivars with necrosis were collected, washed with water, wrapped in aluminium foil, and transported to the Phytopathology and Plant Biotechnology laboratory of WAVE/IFA-Yangambi for analysis (Figure 1). The samples were kept at 4°C in a freezer before analysis.

**Figure 1:** Cassava tuber samples wrapped in aluminium foil and placed in a cooler.

**Table 1:** Different cassava cultivars from which the samples were taken

| Collection fields | Number of samples |
|---|---|
| **WAVE** | 30 |
| **IITA** | 30 |
| **INERA-Yangambi** | 30 |

- Reagents: NaCl (0.85%), Phenylenediamine, Potato Dextrose Agar (PDA), LPGA (Yeast-Peptone-Glucose-Agar), lactophenol, ethyl alcohol 70%, and distilled water.
- Equipment: cooler, optical microscope, microscope with camera, autoclave, water bath, precision balance (to the thousandth pre), hot plate, Bunsen burner, laminar flow hood, and graduated feet. Beaker,

spatula, tongs, plastic box, filter paper, aluminium foil, seed loop, Petri dishes, test tubes, GPS, marker, pen, paper, and so on.

**2.3. Methods**

**2.3.1. Sampling**

A total of 60 samples were obtained, 30 from each cassava collection area (from 6 to 9 months elders).Field observations included data on the highest severity of root necrosis in collection fields from several selected sites.GPS coordinates and cassava variety names were recorded for each sampling location.

Because there were no correlations between root necrosis symptoms and leaf symptoms, only root samples were considered. The seedlings were sampled, uprooted, and their roots counted. Each root was sliced transversely five times at regular intervals along its length.

Each root slice was rated on a scale of 1 to 5 (Hillocks & Thresh, 2000). The degree of root necrosis was determined on each of the five slices as follows:

There are five levels of root necrosis (see figure 2):

- (1) No necrosis roots,
- (2) less than 2%of necrosis,
- (3) from 2 to 5% of necrosis,
- (4) from 5 to 50%of necrosis,
- (5) and more than 50% of necrosis.


Figure 2: severity ratings of cassava root necrosis

**2.3.2. Pathological parameters observed**

$$\textbf{Field incidence} = \frac{\textit{Nnumber of infected plants}}{\textit{Total number of plants observed}} x\ 100 \quad \textbf{(1)}$$

$$\textbf{Lab incidence} = \frac{\textit{Number of petri dishes showing the development of colonies of m.o.}}{\textit{Total number of samples analysed}} x\ 100 \textbf{(2)}$$

Bacterial identification was made by isolating sick cassava tissues in the laboratory using a specialized LPGA culture medium.

**2.3.3. Operating mode**

The procedure for preparing culture media is as follows:

- combine 5g yeast extract, 5g peptone, 10g glucose, 15g bactoagar, and 1000ml distilled water. Homogenize well.
- autoclave for 15-20 minutes at 120°C.
- cool to 50°C in a water bath,
- for the medium into Petri dishes under a laminar flow hood next to a flame (Bunsen burner).
- soak plant tissues in saline solution (NaCl 0.85%).
- spread the suspension by exhaustion on the culture media.
- and incubate for 2 days at 27°C, 85% humidity, pH 7.

**2.2.3.1. Identification**

From a 48-hour pure culture, do an oxidase test (bio Mérieux) to identify the cytochrome oxidase enzyme in the bacteria. This enzyme oxidizes the phenylenediamine reagent to produce indophenol, a purple chemical.

The protocol for the oxidase test is as follows:

- Apply one or two drops of the phenylenediamine reagent on sterile filter paper.

- To identify bacteria, take an aliquot from the yellowish colony on the culture medium and place it on filter paper. Mix it with the reagent using a toothpick.
- After 30 seconds, a reddish tint indicates a positive oxidase test result.

**2.2.3.2. Macroscopic observation**

The emergence of colonies on the surface of a solid medium (nutrient agar) is observed macroscopically (by the naked eye). It enables us to identify the following characteristics: shape, surface appearance, opacity; size, odour, consistency, colour, and/or pigmentation.

Then we did a preliminary Gram stain to determine the sort of grouping, shape, and Gram of the bacteria that comprised each colony.

**2.2.3.3. Microscopic observation**

It was performed immediately after the cells had been stained. It allowed for differentiation based on cell shape (spherical, cylindrical, spiral, coiled, or filamentous), grouping method (chains, asymmetrical clusters, or grafts like Streptococcus), and spore size (Khessid & Kedjedja, 2013).

The morphological properties of the isolates (macroscopic and microscopic) were examined under a microscope. The Commonwealth Mycology Institute (CMI) and Food Microbiology (Guiraud et *al*., 1998) have contributed to the identification of photos collected in the laboratory.

**2.3.4. Experimental setup and statistical analysis**

Because the work was done in the laboratory, the experimental design was fully random blocks, with tuber fragment samples being placed in Petri plates for isolation.

The data from processing the forms were recorded in an Excel file for statistical analysis using the R 4.3.2 program (R Core Team 2023), with the Kruskal-Wallis test at the 5% level.

## III. Results

**3.1. Pathological parameters observed**

Figures 3, 4 and 5 illustrate the occurrences, severity and severity of CRND among various cultivars (or clones) from three collection fields.

**3.1.1. Incidences of CRND**

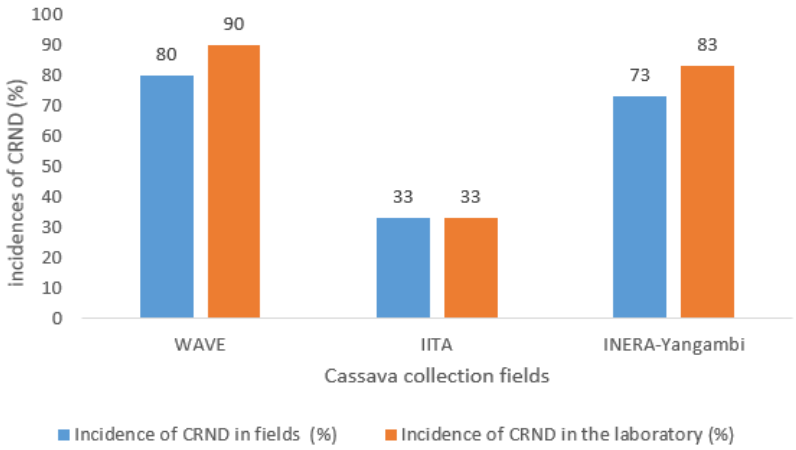


**Figure 3**. Incidences in the field and in the CRND laboratory in three cassava collection fields

Figure 3 shows that the WAVE cassava collection field had 80% field incidence and 90% laboratory incidence (microbial culture). The IITA collection field has a 33% field incidence and an identical laboratory incidence, but INERA-Yangambi has a 73% field incidence and 83% laboratory incidence. The difference between these two pathogenic features (incidence in the field and in the laboratory) is attributable to the presence of fungal and/or bacterial germs in certain ostensibly healthy tuber pieces after culture. The IITA collection field has a low incidence rate since it is a breeding field for improved varieties (Ilona) and the plantation is propagated from healthy cuttings.

The WAVE collection field has an extremely high incidence rate since it collects cassava from all sides, independent of the health state. In addition, it was put in a barren area where cassava production had previously occurred.

Yangambi collection field results from the fact that the collection established contains the same materials several times, revealing a risk of infection in the fields or a loss of the resistant character of these materials.

### 3.1.2. Severity of CRND (scale of 1-5)

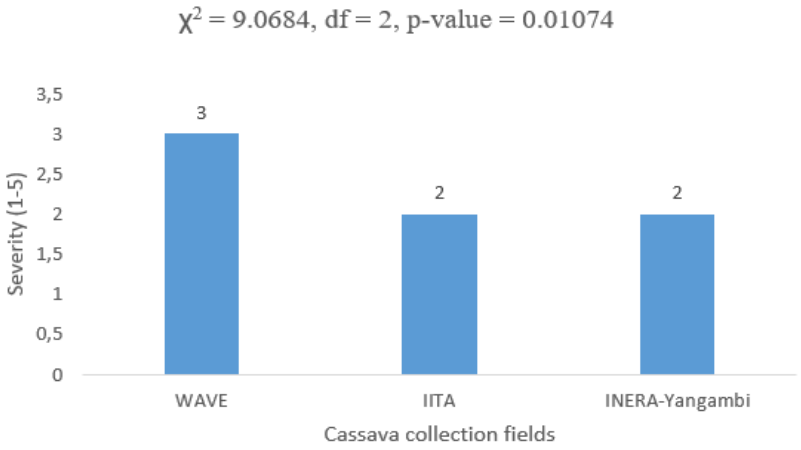


**Figure 4:** Severity of CRND in the three cassava collection fields

Figure 4 reveals that the WAVE cassava collection field has a level 3 CRND severity, whereas the collection field of INERA-Yangambi is level 2, and IITA's is level 2. The Kruskal-Wallis test (df = 2, p-value = 0.01074) at the 5% level reveals a highly significant variation in the severity of CRND across these diverse collection fields.

This difference is because the WAVE collection fields use planting materials from various sources, which may contain pathogens. Additionally, the collection was installed in a fallow area after cassava cultivation a few seasons ago. In contrast, the Yangambi collection field uses the same materials multiple times, which may lead to resistance loss over time. The equipment used at the IITA collection field was carefully selected.

### 3.1.3. Gravity of CRND

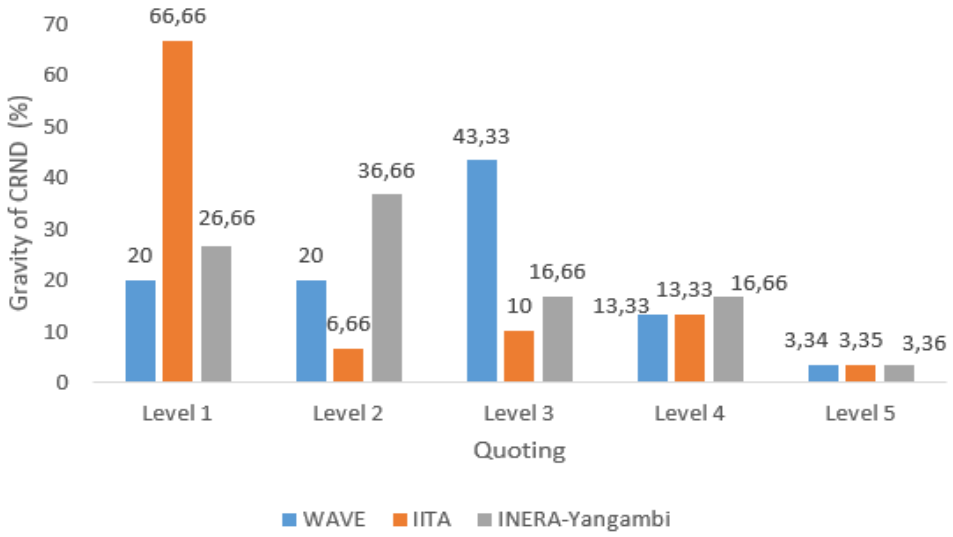


**Figure 5**: Gravity of CRND in the three cassava collection fields

Figure 5 depicts how the gravity of CRND changed based on the place (cassava collection field). We see that more than 60% of cassava plants have a grade of 3 or above in the WAVE collection field, implying that CRND is a severe issue in this area. In the IITA collection field, more than 70% of cassava plants have a rating of less than or equal to 2, indicating that the CRND is less severe. In the INERA-Yangambi collection field, more than 60% of cassava plants have a rating less than or equal to 2, indicating that the CRND is less severe in the latter.

### 3.2. Bacteria isolated and characterised morphologically

#### a. Macroscopic characteristics of the isolates

A total of 90 isolates were isolated from 30 different cultivars from each cassava collecting site. 58 isolates demonstrated growth on LPGA, but 32 isolates did not grow on the same culture medium.

**Table 2:** Macroscopic morphological characteristics of colonies of bacterial isolates on LPGA

| **Collection fields** | **Isolates** | **Colony shape** | **Appearance of the surface of the colony** | **Colony Opacity** | **Colony size** | **Smell** | Growth | Colour |
|---|---|---|---|---|---|---|---|---|
| WAVE | 23 | Rounded and smooth | Viscous | Brilliant | 1.5mm-3mm | Nauseous | Average | Ivory white |
| IITA | 10 | Rounded and smooth | Viscous | Brilliant | 1.5mm-3mm | Nauseous | Average | Ivory white |
| INERA | 25 | Rounded and smooth | Viscous | Brilliant | 1.5mm-3mm | Nauseous | Average | Ivory white |

After purification on LPGA culture medium, bacterial isolates from the Yangambi collection field exhibited similar morphological characteristics, including a rounded shape, viscous appearance, shiny size of 1.5 mm to 3 mm, foul odour, rapid growth, and ivory white colour. Additionally, 23 isolates from the WAVE collection field and 10 isolates from the IITA collection fields exhibited the same morphological characteristics.

The numerical disparity in the INERA-Yangambicollection field is due to the existence of several cassava clones under study.In the WAVE collection field, the cuttings come from many areas of the Democratic Republic of Congo, and they might contain specific illnesses that, once established, can present themselves.The cuttings are planted in a fallow area that has previously been used for cassava farming. The soil may contain disease germs that have been passed down to the cuttings.

The low IITA collection field result in comparison to that of WAVE is because the cuttings from this field were carefully selected to resist certain cassava diseases, and the field in which these cuttings were installed is from the clearing of a secondary forest, which does not have a cultural precedent capable of leaving certain cassava disease germs in its soil.

#### b. Microscopic characteristics of the isolates

The microscopic examination focused on the identification of the typical structures of the thirty-three bacterial isolates collected. Cell form, method of grouping, spore presence, size, and Gram staining were all seen under a microscope with a 40x magnification camera (1024x768). The findings collected are compiled in the bellow table.

**Table 3:** Microscopic morphological characteristics of bacterial isolates on LPGA

| **Collection fields** | **Isolates** | **Cell shape** | **Grouping mode** | **Presence of spores** | **Size** | **Colouring gram** |
|---|---|---|---|---|---|---|
| WAVE | 23 | As shells or small sticks | Isolated or asymmetric cluster | No spores | 0.6-1.5 µm | Gram- |
| IITA | 10 | As shells or small sticks | Isolated or asymmetric cluster | No spores | 0.6-1.5 µm | Gram- |
| INERA | 25 | As shells or small sticks | Isolated or asymmetric cluster | No spores | 0.6-1.5 µm | Gram- |

After examining the macroscopic and microscopic morphological characteristics of various isolates (tables 2 and 3), the results of morphological characterisation of the isolates assigned 25 isolates from the INREA collection field, 23 isolates from the WAVE collection field, and 10 isolates from the IITA collection field to the genus Xanthomonas. This discrepancy in results across three collection fields may be explained by evaluating the data in Table (2) above.

## IV. Discussions

Few research has been conducted on cassava root necrosis disease, which is common in specific parts of the Democratic Republic of Congo. Cassava root necrosis disease is like CBSD in that it causes root necrosis but shows different signs on the stem and foliage.

The CRND was highly severe, with an incidence of 80% in the WAVE collection fields, since the planting materials came from all sources and most likely included pathogen germs, but also because the collection was built in a fallow area that had previously been cultivated with cassava. This cultural antecedent

was the local cassava varieties that farmers utilized in their various fields. Unfortunately, these materials frequently do not go through phytosanitary testing before being installed.

At the INERA-Yangambi collection field, CRND was less severe, with a 73% incidence. This effect is explained by the fact that the collection consists of the same materials put numerous times in the same spot, resulting in a possible loss of resistance over time.

In the IITA collection field, CRND was less severe, with a disease incidence of 30%. This is because the materials were chosen based on their health state, and the collection was built on ground that had not previously been grown with cassava. SENASEM performed Phyto sanitation and validation of seed standards.

Knowledge of disease aetiology is critical in the battle against plant diseases (Twumasi et *al*., 2014).

The selection of an LPGA medium for the isolation of bacterial species is based on its usage in prior research and is suited for the isolation of a wide spectrum of bacteria (Grousson et *al*., 1990).

The bacteria that cause root necrosis disease were identified using 90 isolates. The morphological identification findings indicated 58 isolates of the species Xanthomonas on all samples from the three cassava collection areas (WAVE, IITA, and INERA).

Amuri's (2022) morphological characterization of the isolates identified 17 isolates from the Fusarium genus. Furthermore, molecular analysis indicated a high similarity between these isolates and the species *Fusarium solani*.

Nyaka et *al*. (2015) isolated and identified certain pathogenic fungi that cause cassava root rot disease (*Manihot esculenta* Crantz) in Cameroon. They collected 20 isolates from 64 cassava stems that showed indications of root rot and discovered seven fungi, including *Colletotrichum sp., Fusarium sp., Pestalotia sp., Geotrichum sp., Trichoderma viride*, and *Botryodiplodia theobroma*.

During the HTS sequencing performed by the team of Bakelana (2022), reads were attributed to microorganisms, including bacteria from the genus *Xanthomonas* and fungus from the genus *Fusarium*.Bakelana's investigation partially verifies our findings about the possible presence of bacteria from the genus *Xanthomonas* in samples exhibiting signs of cassava root necrosis.Unfortunately, no previous research has been able to link this to the root necrosis reported in cassava.

## V. Conclusion

The goal of this study was to identify the bacteria that cause cassava root necrosis disease (CRND) in the Tshopo area of the Democratic Republic of Congo.

To do this, it was required to first evaluate the severity of CRND in the cassava collection fields of WAVE-IFA / Yangambi, IITA, and INERA-Yangambi, and then to identify the bacteria responsible for the disease.

The CRND data revealed that at the WAVE-IFA / Yangambi collection field, the incidence was 80% with a severity of 3, whereas at the INERA- Yangambi and IITA collection fields, it was 73% and 33%, respectively, with a severity of 2 for each. The Kruskal-Wallis test at the 5% level (df = 2, p-value = 0.01074) reveals substantial variations in the CRND severity of these three collection fields.

The severity of CRND in these collection fields is as follows: the illness is too bad in the WAVE collection field, but less serious in the other two.

The morphological analysis (macroscopic and microscopic) of the isolates on the LPGA yielded 25, 23, and 10 isolates of the genus *Xanthomonas* from the INERA-Yangambi, WAVE, and IITA collections, respectively.

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