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Methodology proposal for isolating fungi from *Manihot esculenta* Crantz storage roots with rot symptoms

Propuesta metodológica para el aislamiento de hongos a partir de raíces almacenadoras de *Manihot esculenta* Crantz con síntomas de pudrición

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Abstract
Cassava (*Manihot esculenta Crantz*) is a vital staple food for millions of people worldwide, primarily valued for its edible storage roots. However, these roots are vulnerable to Cassava Root Rot Disease (CRRD), often caused by fungal pathogens such as *Fusarium*, *Lasiodiplodia*, and *Phytophthora*. This disease poses a significant threat to food security in tropical and subtropical regions. Accurate microbial identification is essential for understanding CRRD pathology. However, conventional methods for isolating microorganisms from diseased roots—particularly fungi—have limitations. For instance, incomplete surface sterilization can lead to the isolation of non-axenic fungal colonies. To address this issue, we propose a modified methodology that includes a surface particle-removal step and sterility controls to validate its effectiveness. Our method significantly improved the isolation of axenic fungal colonies from CRRD-affected roots compared to traditional techniques across three different culture media. Out of 60 samples per method, the proposed method yielded a statistically higher proportion of axenic fungal colonies (88.3%) than the traditional approach (30%). Although the proposed method facilitates microbial isolation, further validation is needed to assess its reliability across various pathogens and environmental conditions.

Keywords: Cassava; Isolation; Microorganisms; Root rot disease; Root sample.

Resumen
La mandioca (*Manihot esculenta* Crantz) es un alimento básico para millones de personas en todo el mundo, principalmente debido a sus raíces almacenadoras comestibles. Sin embargo, dichas raíces son susceptibles a la Pudrición Radical de la Mandioca (PRM), enfermedad causada frecuentemente por hongos (*Fusarium*, *Lasiodiplodia* o *Phytophthora*), que es una amenaza para la seguridad alimentaria en regiones tropicales y subtropicales. Una identificación precisa de los microorganismos involucrados es fundamental para mejor comprensión de la PRM. En este sentido, los métodos convencionales de aislamiento de microorganismos a partir de raíces enfermas, en particular hongos, presentan ciertas limitaciones. Por ejemplo, una incompleta esterilización superficial de las muestras puede conducir al aislamiento de colonias fúngicas no axénicas. En este trabajo, se propone una metodología modificada, que incorpora un paso de remoción de partículas superficiales y controles de esterilidad que permiten validar la eficacia de esta etapa. Nuestro método mejoró significativamente el aislamiento de colonias fúngicas axénicas a partir de raíces con síntomas de PRM en comparación con las técnicas tradicionales en tres medios de cultivo distintos. De 60 muestras procesadas por cada método, la metodología propuesta logró un porcentaje estadísticamente superior de colonias fúngicas axénicas (88.3%) en comparación con el enfoque tradicional (30%). A pesar de que el método propuesto facilitó el aislamiento microbiológico, se requiere una validación adicional para evaluar su robustez frente a distintos patógenos y condiciones ambientales.

Palabras clave: Mandioca; Aislamiento; Microorganismos; Pudrición radical; Muestra de raíz.

Introduction

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae family, is a perennial shrub (1) cultivated in tropical and subtropical regions (2,3). Its storage roots (SRs) serve as a staple food in South and North America, Central Africa, and Southeast Asia (4). In 2021, global cassava production reached 315 million tonnes, with a total trade value of 3.65 billion USD (5).

The anatomical structure of the SR comprises three distinct layers: the peel, pulp, and central fibers (6). The peel includes both the periderm, located on the external surface, and the cortex layer (6,7). The pulp, which constitutes the edible portion, is mainly

composed of starch-rich secondary xylem tissue (7). At the core, the central fibers contain rows of parenchyma and xylem sclerenchyma vessels (8).

The productivity and quality of storage roots (SR) can be severely affected by various diseases, including Cassava Root Rot Disease (CRRD). CRRD leads to root necrosis, which alters the consistency and appearance of the edible pulp and results in yield losses ranging from 30% to 100% (9). It is frequently caused by microbial pathogens, particularly fungi such as *Fusarium*, *Neoscytalidium*, *Lasiodiplodia*, and *Phytophthora* (10–12).

Understanding the diversity and characteristics of microorganisms associated with CRRD is crucial for developing effective disease management strategies (9,10,13). As noted by Hochmuth et al. (2012), the examination of plant tissue supports both nutrient monitoring and diagnostic efforts. Another important objective is to determine the microbial composition within and surrounding the plant tissue (15). Diagnosing microbial pathogens involves identifying both the causative agent of the disease and its associated characteristics and symptoms (15). The search for an optimal technique to investigate root–microorganism interactions, particularly those involving fungi, remains a significant challenge (16,17).

Microbial pathogen diagnosis involves identifying the causative agent of a disease, along with its characteristics and symptoms (15). In particular, diagnosing microbial pathogens in roots presents additional complexity compared to detection in other plant tissues. Roots are embedded in the soil matrix, where they host a diverse community of microorganisms (18,19), and they engage in early interactions with both the soil and its microbial inhabitants (18,19). Traditional methods for isolating fungi from plant tissues, including storage roots (SR), typically begin with the removal of visible soil debris by rinsing with tap water (21), followed by dissection. The resulting root pieces are then surface-sterilized and cultured to isolate microorganisms (15).

During microbial isolation from decayed root samples, a major limitation is the practical impossibility of removing all soil particles using only water rinsing (22–24), particularly those lodged within or between root tissues (19,25,26). As a result, cleaning—specifically the separation of debris—becomes a complex and labor-intensive task (21). Residual soil particles contribute a substantial microbial load (27), increasing the likelihood of mixed microbial cultures and thereby complicating both isolation and analysis (23). Although sequential sterilization steps are intended to reduce microbial contamination, they do not always ensure complete asepsis. Addressing the challenges of microbial diagnosis in CRRD requires the development of novel procedures that retain consensus protocols while remaining efficient and practical (28). To overcome some of these limitations and enhance the qualitative isolation of microorganisms from storage roots (SR) showing CRRD symptoms, we proposed a modified isolation method. This method built upon the traditional workflow by incorporating an additional surface-particle removal step, thereby increasing the probability of obtaining axenic fungal colonies.

Two Sterility Controls (SCs) were included to evaluate the effectiveness of the surface-particle removal step: surface imprinting onto culture media (SC1) (29–31) and cultivation of an aliquot of the final wash water after mechanical agitation (SC2) (32–34). SC1 involved transferring potential surface microorganisms onto the agar by pressing the root sample directly onto the medium surface. Imprinting is suitable for various surface types, including smooth, rough, and irregular textures (35). SC1 was critical in confirming that the isolated fungi were not present on the surface of the sample. Similarly, SC2 confirmed the absence of microbial load detaching from the sample surface, thereby verifying the effectiveness of the washing process. Together, SC1 and SC2 served as baseline controls to differentiate microorganisms introduced during handling.

Materials and methods

Collection of the CRRD samples

Field collection was carried out in accordance with local regulations governing access to natural and genetic resources in the province of Misiones. Prior informed consent was obtained from the Misiones Institute of Biodiversity (Resolution No. 44/24). A total of 20 storage roots (SR) exhibiting CRRD symptoms (36) were collected from a cassava

field near the town of Wanda, Misiones, Argentina (25º 95′ S; 54º 52′ W), where the soil is classified as Ultisol (37). The SR were stored at 18 ± 2 ºC in plastic bags for less than 24 hours (21) before arrival at the laboratory.

Description of the isolation methods tested

In this study, two isolation methods were evaluated: the traditional method (A) and the proposed method (B) (Fig. **1**). Method A was adapted from Nyaka et al. (2015) (38), Oliveira et al. (2016) (39), and Sangpueak et al. (2023) (13), and included the following steps: (I) Cleaning: removal of soil debris using tap water. (II) Dissection: cutting the roots into small pieces (approximately 5–25 mm). (III) Sterilization: sequential treatment of the root pieces with 70% ethanol, followed by 1% sodium hypochlorite, and finally 90% ethanol, for 1 minute each. (IV) Inoculation: placing the root sample in a sterile 90 mm Petri dish containing 20 mL of culture medium. (V) Cultivation.

Method B consisted of the following steps: (I) Cleaning: removal of visible soil debris using tap water. (II) Dissection: cutting the roots into small pieces (approximately 5-25mm) differentiating pulp-only samples. (III) Sterilization: sequential treatment with 70% ethanol, 1% sodium hypochlorite, and 90% ethanol, for 5 minutes each. (IV) Surface-particle removal: agitating the root pieces in an analog vortex at medium speed for 3 minutes (34,40) in tubes containing sterile distilled water (SDW). The samples are then transferred to new tubes with fresh SDW, and the step is repeated until no sediments are released after agitation. The number of washes should be first guided by visual clarity of the wash water and then confirmed using the sterility controls (SCs). (V) Inoculation: for each sample, inoculate 20 µL of the water from the final rinse (SC2), perform the imprinting technique (SC1), and place the root piece in a sterile 90 mm Petri dish containing 20 mL of culture medium. (VI) Cultivation. For the investigation of potential CRRD causal agents, only samples yielding axenic microbial colonies should be considered. In Method B, this condition is confirmed by the absence of microbial growth in both sterility controls (see Case 1 in Fig. **2**).

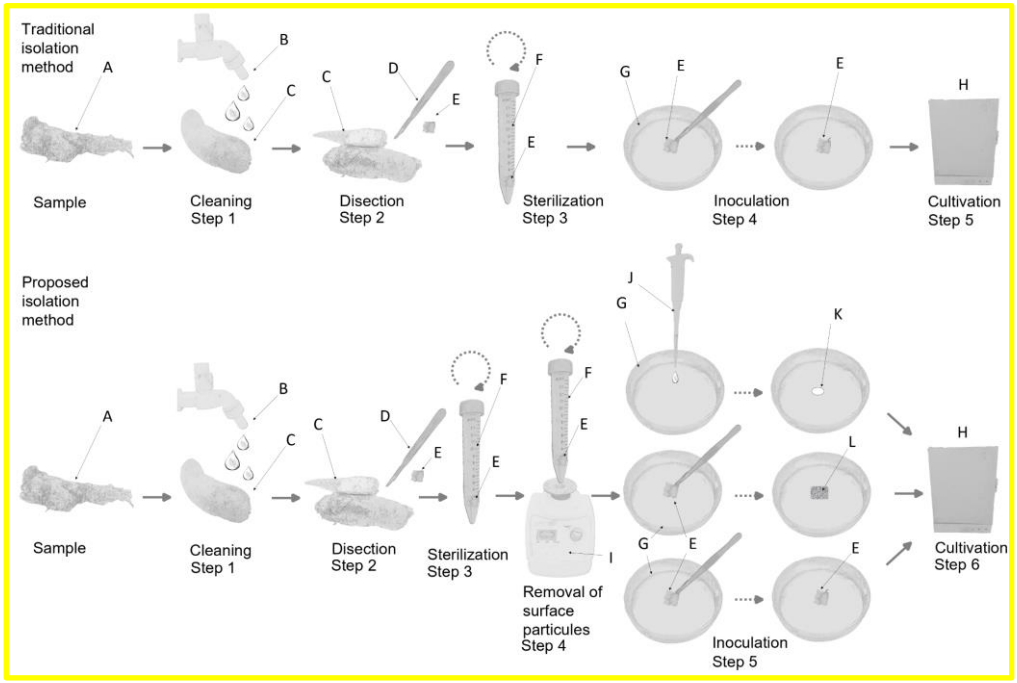

Fig. 1 Graphical summary of the traditional and proposed isolation methods. A= cassava storage root embedded with soil. B= tap water. C= cassava storage root. D= bistoury. E= cassava storage root sample. F= centrifuge tube. G= Petri dish. H= incubator. I= analog vortex. J= micropipette. K= water from the last wash. L= imprinting.

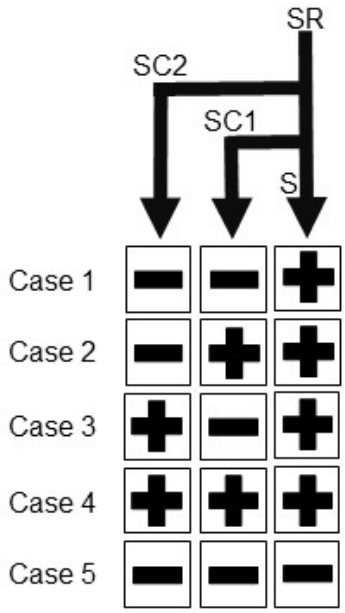

Fig. 2 Schematic representation of possible outcomes in the proposed method. SR= storage root. S= dissected root sample. SC1= sterility control 1 (imprinting). SC2= sterility control 2 (water from the last wash). The symbols **+** and **-** indicate microbial growth and absence of microbial growth, respectively. Case 1: microbial growth is observed only in **S**. Case 2: microbial growth is observed in **S** and **SC1**. Case 3: microbial growth is observed in **S** and **SC2**. Case 4: microbial growth is observed in **S**, **SC1**, and **SC2**. Case 5: no microbial growth is observed.

Calibration of the number of washes (step IV)

To determine the optimal number of washes during the surface particle removal step, four washing frequencies were tested: 0 washes (Level 1), 1 wash (Level 2), 3 washes (Level 3), and 5 washes (Level 4). For this experiment, a total of 80 SR samples were cultivated, with 20 samples allocated to each level. The samples were placed in sterile 90 mm Petri dishes containing 20 mL of PDA and incubated in darkness at 28 ± 2 °C for 7–10 days. Following the cultivation period, the colonies were classified into two categories: axenic samples, containing only one type of fungal colony, and non-axenic samples, containing two or more types of fungal colonies (Fig. **3** and Fig. **4**).

For the statistical analysis, the number of washes was considered the independent variable, and the classification of fungal colonies was the dependent variable. Level 1 (no surface-particle removal, as in the traditional method) was used as the reference category. The levels were coded as 0, 1, 3, and 5, corresponding to the number of washes in each treatment. The assumptions of homogeneity across levels and a linear trend were assessed using the chi-square (χ^2) test at a significance level of 0.05, using Epidat v3.1 (41).

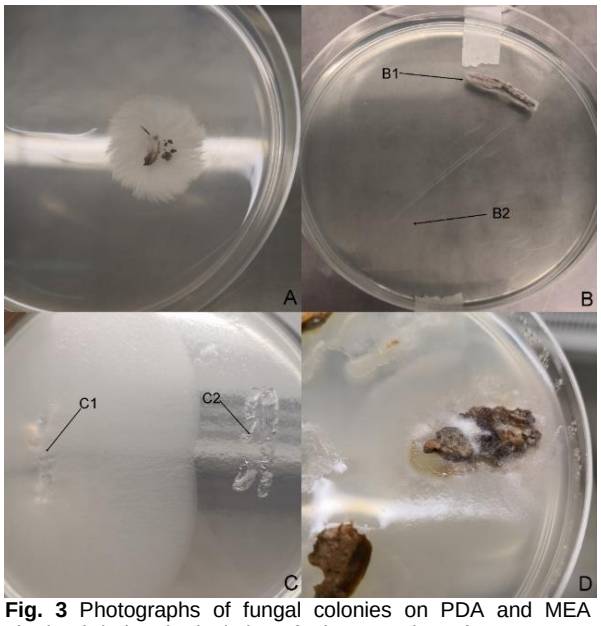

Fig. 3 Photographs of fungal colonies on PDA and MEA obtained during the isolation of microorganisms from cassava root rot samples. A= axenic colony. B= axenic colony (B1) with no microbial growth in the imprinting (B2). C= Imprints with (C1) and without (C2) microbial growth. D= sample showing more than one type of microbial colony (non-axenic).

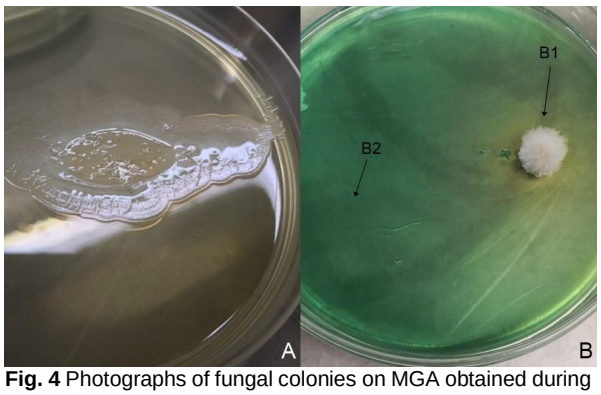

Fig. 4 Photographs of fungal colonies on MGA obtained during the isolation of microorganisms from cassava root rot samples. A= Imprint with microbial growth on MGA. B= axenic colony (B1) with no microbial growth in the imprinting (B2) on MGA.

Comparison in the proportion of axenic fungal colonies between the isolation methods

The traditional method (A) and the proposed method (B, with the optimal number of washes) were tested using a stratified design. Three types of culture media were used: Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Malachite Green Agar (MGA), the latter being a selective medium for *Fusarium* species (42). A total of 120 SR samples were cultivated, with 60 samples processed using method A and 60 using method B. For each method, the 60 samples were evenly distributed across the three

media types, with 20 samples per medium. The samples were placed in sterile 90 mm Petri dishes containing 20 mL of the corresponding culture medium (PDA, MEA, or MGA) and incubated in darkness at 28 ± 2 ºC for 7–10 days. Following the incubation period, the fungal colonies from each sample were classified as previously described—either axenic or non-axenic (Fig. **3** and Fig. **4**). For the statistical analysis, two independent variables (methods A and B) within three strata (PDA, MEA, and MGA) and one dependent variable (classification of fungal colonies) were considered. Homogeneity across strata was assessed using the X^2 (chi-square) test with a significance level of 0.05. To evaluate potential statistically significant differences between the two isolation methods in the proportion of axenic fungal colonies isolated from SR with CRRD symptoms (i.e., association across strata), the Mantel-Haenszel test was applied, also with a significance level of 0.05, using Epidat v3.1 (41).

Results

Preliminary calibration of the proposed method

It was observed that relatively small samples (approximately 25 mm or less) were optimal for isolation. Preliminary data showed that all samples exhibited microbial growth following a 5-minute duration for each sterilization step. However, some samples did not produce microbial growth with extended sterilization times (see case 5 in Fig. **2**). Therefore, a sterilization time of 5 minutes was deemed sufficient for this step. Additionally, different numbers of samples per Petri dish were tested preliminarily. Up to 2–3 samples per dish were manageable, considering a cultivation period of 7–10 days. Optionally, samples in a single Petri dish could include those from tissue incubation, imprinting, and the water from the last rinse. Fungal growth was apparent from the 2nd day of cultivation, becoming more vigorous by day 10. After this period, most microbial colonies began to overlap.

Calibration of the number of washes (step IV)

Results indicated a statistically significant difference ($P < 0.01$) in the proportion of axenic colonies across the levels studied (0 to 5 washes), with a clear linear trend ($P < 0.001$). The highest proportion of axenic fungal colonies (85%) was observed with five washing repetitions, while the lowest (25%) was recorded with no washing (Fig. **5**). Based on these findings, five washing repetitions (level 4) were selected for use in the proposed method in subsequent analyses.

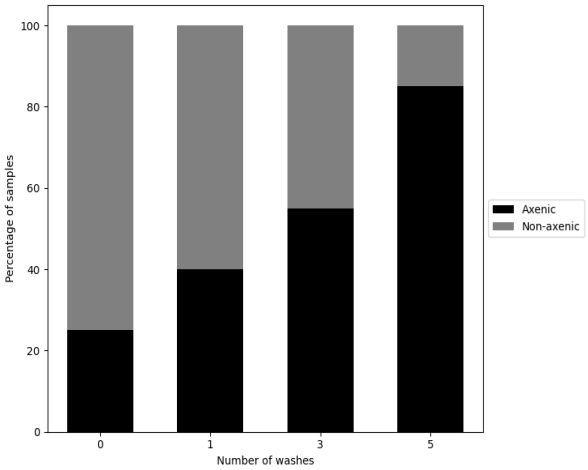

Fig. 5 Proportion of axenic (black bars) and non-axenic (grey bars) fungal cultures obtained from SR samples subjected to varying numbers of washes during the surface-particle removal step.

Comparison in the proportion of axenic fungal colonies between the isolation methods

Results indicated a statistically significant difference ($P < 0.001$) in the proportion of axenic colonies between the two isolation methods (Fig. **6**). Overall, Method A yielded 18 axenic colonies out of 60 samples (30%), whereas Method B yielded 53 axenic colonies (88.3%). No statistically significant differences were observed across culture media strata ($P > 0.5$); however, a significant association between the isolation method and the proportion of axenic colonies was detected after adjusting for culture medium using the Mantel-Haenszel test ($P < 0.001$). Therefore, results can be analyzed as a whole (independently of strata), indicating that the type of culture media did not significantly influence the proportion of axenic fungal colonies. In other words, the observed differences were attributable to the isolation method used. Notably, non-axenic colonies obtained with Method B also exhibited microbial growth in both sterility controls.

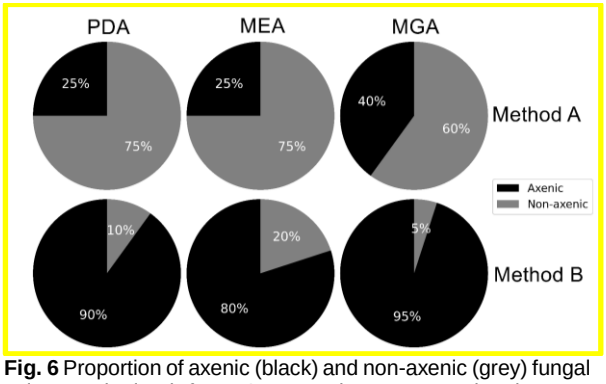

Fig. 6 Proportion of axenic (black) and non-axenic (grey) fungal cultures obtained from SR samples processed using two isolation methods (A and B) across three different culture media: potato dextrose agar (PDA), malt-extract agar (MEA) and malachite green agar (MGA).

Discussion

In the proposed method for isolating fungi from SR with CRRD symptoms (method B), specific improvements were introduced compared to the traditional approach (method A). Both methods begin with the same initial steps (steps I-III: cleaning, dissection and sterilization). The order of steps II and III was carefully considered. Although reversing the sequence is possible, it is recommended to perform dissection before sterilization. This sequence allows for more precise and localized sterilization, potentially reducing both the required waiting time and the amounts of sterilizing agents used.

During dissection (step II), the size and selection of the samples should be considered. In this experiment, samples were cut specifically to target the pulp tissues, as it is the main component of the root where symptoms are observed. However, further differentiation between tissue types may become crucial for understanding fungal distribution within the root. Although the optimal size for fungal recovery requires further evaluation, preliminary experiments showed that even small samples contained a high microbial load.

Although both methods used ethanol and sodium hypochlorite solutions for sterilization (step III), it is important to assess whether these agents are the most effective at eliminating all potential surface contaminants. Further studies could explore alternative disinfectants or variations in the concentration and duration of the disinfection process to optimize sterilization (43), while introducing none or only minimal artifacts to the samples. Our results indicated that the removal of surface soil particles (step IV) played a crucial role in improving sample cleanliness, thereby reducing the risk of microbial contamination during subsequent processes. Additionally, two sterility controls were included for this step. Ideally, these sterility controls should show no microbial growth, indicating the absence of contaminants on the sample surface. Consequently, if microbial growth is observed only in the root sample, it can be inferred that the microbes originated exclusively from inside the sample and not from its surface (see case 1 in Fig. **2**).

In case of severe necrosis where tissue differentiation is impossible, it would be unclear which root tissue the fungi originated from. However, even in such cases, the proposed method may reduce the likelihood of obtaining isolates from external sources (e.g., soil particles attached to the roots), due to validation through SCs.

More than five agitation cycles may be necessary in step IV when samples are highly decayed or damaged (44). This is supported by the observation that non-axenic colonies obtained with method B also showed fungal growth in SC1 and SC2. However, it is important to note that axenic colonies exhibiting growth in the sterility controls, but with morphology consistent with the colony from the pulp sample, may still be relevant in the context of CRRD (see cases 2, 3 and 4 in Fig. **2**), although their exact tissue origin cannot be determined (16).

The removal of surface soil particles could be further improved by substituting SDW with alternative solutions (23). Evidence indicates that chelating agents, detergents, or saline solutions enhance particle detachment (24). Incorporating such solutions into the agitation process can improve the removal of surface contaminants, leading to cleaner and more reliable samples for microbial isolation. Additionally, exploring alternative mechanical agitation techniques (24) can further optimize surface soil particle removal in method B. For instance, Cuske *et al.* 2014 (45) demonstrated the effectiveness of ultrasonic agitation for cleaning plant samples. However, it should be noted that these techniques might damage fragile cells (22), warranting further investigation.

Exploring different incubation conditions could offer valuable insights into enhancing the growth and differentiation of fungal colonies during the isolation process. In this

study, incubation was carried out under standard conditions: in darkness at a temperature of 28 ± 2 ºC for 7-10 days. However, varying incubation temperatures (20,40,46) or durations could be investigated to optimize the isolation procedure.

Regarding the culture media, this study utilized PDA and MEA —both non-selective, nutrient rich media commonly employed for fungal isolation of cassava SR samples with CRRD symptoms (47)—as well as the selective medium MGA. However, it would be valuable to investigate whether isolation efficiency could be improved by incorporating inhibitors such as antibiotics (39) or specific nutrients into the growth media (40,43). Testing different media types (16), including additional selective or general-purpose media that support the growth of a wide range of fungi, could provide further insights into the diversity and composition of the fungal community associated with CRRD.

Ensuring quality control during the isolation process is essential for obtaining accurate and reliable results. Visual inspection, as suggested by Zhu *et al.* (2008) (48), can complement the proposed method by serving as an additional checkpoint for contamination. Moreover, incorporating a mechanical purification step on agar plates —such as the hyphal tip technique (15) or the method proposed by Shi *et al.* (2019) (49)—can further validate sample cleanliness.

Regarding the practical applicability and reproducibility of the proposed method, it was developed by introducing minor modifications to the widely used protocol for isolating fungi from SR (11,12), making it easily implementable across various laboratory settings. The central idea behind the proposed method was to maintain practicality and accessibility for researchers familiar with conventional techniques, as recommended by Fischer and Zigmond (2010) (28). Although the proposed method slightly increases the complexity of the process —requiring relatively more resources and time during the isolation of microorganisms from CRRD samples—this investment is offset by a reduced risk of handling non-axenic fungal colonies in subsequent steps and the achievement of more consistent results (50).

The wide-ranging assortment of cultivable cassava varieties and their cultivation across diverse soil profiles (24) highlights the need for a versatile approach to investigating CRRD, emphasizing the importance of accounting for the heterogeneous nature of the disease (10). Different forms of CRRD can be caused by various pathogens, each requiring a tailored investigative strategy. In this context, the necessity of cross-validating the proposed method—considering different types of pathogens and field conditions—is evident. Moreover, it is important to recognize that CRRD may involve microbial succession throughout the progression of the disease (51), meaning that the diagnostic perspective could shift depending on the specific timing of sample collection.

Sharing practical tips and addressing potential challenges encountered during microbial isolation from plant tissue could support the successful adoption of more advanced methods by researchers (28,52). Additionally, discussing practical limitations—such as the time, resources, and expertise required to implement the method in larger-scale studies— would be beneficial (53).

Conclusions

The proposed method resulted in a statistically significant increase in the proportion of axenic colonies isolated from cassava SR samples with CRRD symptoms, offering a more systematic approach to mitigating the effects of incomplete surface sterilization. This method has the potential to contribute to a more precise understanding of the microbial dynamics involved in CRRD.

List of abbreviations

CRRD= Cassava Root Rot Disease
MEA= Malt-Extract Agar
MGA= Malachite Green Agar
PDA= Potato Dextrose Agar
PRM= Pudrición Radical en Mandioca
SC= Sterility Control
SDW= Distilled Sterilized Water
SR= Cassava Storage Roots

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Data availability statement

All data generated during this study are included in this published article.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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