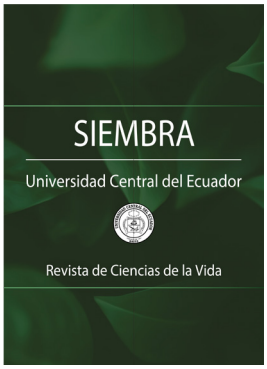
Isolation and pathogenicity of associated agents to root rot disease in cocoyam (Xanthosoma violaceum L. Schott) in Nicaragua

Aislamiento y patogenicidad de agentes asociados al mal seco en quequisque (Xanthosoma violaceum L. Schott), en Nicaragua



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Abstract

Root rot represents the most destructive disease of cocoyam (*Xanthosoma violaceum)*. The isolation of *Pythium myriotylum* requires the use of fresh root samples, so specific isolation techniques and selective growth media must be developed. The objective of this study was to evaluate techniques for the isolation of *Pythium myriotylum* and to determine the pathogenicity of microorganisms associated with root rot in cocoyam. The efficacy of three isolation methods was assessed: a) soil dilutions, b) roots exhibiting symptoms of root rot, and c) cocoyam leaves as baits [HQC]. The isolates were placed in different culture media, namely a) potato dextrose agar, b) water agar + streptomycin sulfate, c) V8 + Pimaricin, Ampicillin, Rifampicin and Benomyl [PARB]; and d) tetrazolium chloride medium, for bacteria. The isolated microorganisms were identified using taxonomic keys. The pathogenicity of the isolates was evaluated in a greenhouse setting using with vitroplants of cocoyam Lila. This was done by inoculating the isolates obtained, alone and in combination, to a sterile substrate and a control treatment without inoculation. The *Pythium* spp. demonstrated mycelial growth with an abundance of fruiting structures when the HQC method and V8-PARB culture media were employed. The pathogenicity test revealed that the combined treatments with *P. myriotylum* resulted in the manifestation of symptoms indicative of root damage. The combination of soil isolation with cocoyam leaves, and the V8-PARB culture media was the most effective method for the isolation of *Pythium myriotylum.* This approach resulted in the growth of abundant mycelium that facilitated morphometric identification. *P. myriotylum* was observed to indice symptoms consistent with root rot in vitroplants of cocoyam. Inoculation of plants with *F. solani* and *R. solanacearum,* either individually or in combination, did not result in the manifestation of symptoms characteristic of root rot.

Keywords: *Pythium myriotylum*, infectious capacity, infective agents, pathogenicity test.

Resumen

El mal seco es la enfermedad más destructiva del quequisque (*Xanthosoma violaceum*). El aislamiento de *Pythium myriotylum* requiere de mues-

tras de raíces frescas; por lo que se deben desarrollar técnicas de aislamiento específicas y medios selectivos. En este estudio, se evaluaron técnicas para el aislamiento de *Pythium myriotylum* y se determinó la patogenicidad de microorganismos asociados a mal seco en quequisque. Se evaluaron tres métodos de aislamiento: a) diluciones de suelo, b) raíces con síntomas de mal seco, y c) hojas de quequisque como cebos [HQC]. Se colocaron los aislados en diferentes medios de cultivo a) papa dextrosa agar, b) agar agua + sulfato de estreptomicina, c) V8 + Pimaricina, Ampicilina, Rifampicina y Benomil [PARB]; y d) el medio cloruro de tetrazolio, para bacterias. Se utilizaron claves taxonómicas para la identificación de los microorganismos aislados. La patogenicidad se evaluó en invernadero con vitroplantas de quequisque Lila al inocular los aislados obtenidos, solos y su combinación, al sustrato estéril y un tratamiento testigo sin inoculación. Agentes patógenos como *P. myriotylum*, *F. solani* y *R. solanacearum*, fueron aislados. *Pythium* spp. tuvo un crecimiento micelial con estructuras fructíferas abundantes con el método HQC en V8-PARB. En la prueba de patogenicidad, los tratamientos combinados con *P. myriotylum* mostraron síntomas de afectación en las raíces. El método de aislamiento de *Pythium myriotylum* a partir de suelo, con hojas de quequisque y combinado con el medio de cultivo V8-PARB fue el más efectivo, obteniéndose aislados con micelio abundante que facilitaron su identificación morfométrica. *P. myriotylum* provocó síntomas característicos de mal seco en las vitroplantas de quequisque lila. Las plantas inoculadas con *F. solani* y *R. solanacearum,* solos o combinados, no presentaron síntomas de mal seco.

Palabras clave: *Pythium myriotylum,* capacidad infectiva, agentes infecciosos, prueba de patogenicidad.

1. Introduction

Root rot is the most destructive disease in cocoyam (*Xanthosoma* spp.) at global scale (Saborío et al., 2004). It can cause between 70 to 80% yield loss (Folgueras Montiel et al., 2015) or 100% (Acebedo Rivera & Navarro Matute, 2010; Saborío et al., 2004). The symptoms can be slow growth, chlorosis, and necrosis of old leaves; light-green colored young leaves, and curved leaves with reduction or absence of the root system in severe cases (Reyes Castro, 2006; Reyes Castro et al., 2013). The pathogen persists in the soil for years and once it is infested, it cannot be farmed again due to the survival capacity of the pathogen in the soils (Saborío et al., 2004).

In Nicaragua, root rot is present in productive zones for exportation (Nueva Guinea, El Rama, Río San Juan) and in no traditional zones of the Central Region at the agricultural border of the humid tropic, where good yield is obtained for no more than two consecutive cycles. This problem makes farmers leave the zone to continue the production in disease-free soils, which makes production more expensive and pollutes the pathogen-free soils (Saavedra & Reyes, 2012).

Different pathogens have been associated to root rot: *Rhizoctonia*, *Pythium*, *Erwinia* and *Pseudomonas* (Giacometti & León, 1994), *Sclerotium rolfsii* (Bejarano-Mendoza et al., 1998); *Fusarium* spp. (Saborío et al., 2004) and complex fungi in Cuba (Folgueras Montiel et al., 2015). Other studies reported *Pythium myriotylum* Dreschler as the only agent of the disease (Nzietchueng, 1984; Pacumbaba et al., 1992; Perneel et al., 2006, Tambong et al., 1999).

In Nicaragua, *P. myriotylum* was isolated from root cuts that had recent symptoms of the disease. The isolation was made in the agar culture medium with corn flour plus antibiotics pimaricin, ampicillin, and rifampicin (Rodríguez Zamora & Ramírez Reynoza, 2012). Other authors isolated the pathogen from roots that contained stained or necrotic sections in agar media with antibiotics (streptomycin) (Olorunleke et al., 2014; Perneel et al., 2006; Tambong et al., 1999).

The methodology used in Nicaragua to isolate *P. myriotylum* requires fresh tissue samples with recent lesions. Phytopathology labs are located at more than 250 km of the productive zones of cocoyam, which makes sampling of fresh roots difficult. Therefore, PDA medium without antibiotics is used to collect samples, but this promotes the growth of other fungi that could inhibit the growth of *P. myriotylum*. This makes it difficult to obtain a pure isolate for pathogenicity testing and other studies. Other more practical methods and culture media are thus needed to isolate *P. myriotylum* from infested soil, without the need for living plants. Some studies reported the use of baits from different plant parts to capture the oomycetes from the soil, such as carnation petal (*Dianthus caryophyllus* L.) and chiltoma fruit (*Capsicum annum* L.) baits (Sinobas et al.,1999; Urrutia-Anaya & Pacheco-Aguilar, 2009).

This study aimed to evaluate isolation methods for *Pythium myriotylum* in lila cocoyam (*Xanthosoma violaceum*) and other microorganisms associated to root rot. It also aimed to perform pathogenicity tests to verify the causal agent of the disease.

2. Materials and Methods

2.1. Sampling

Five four-months-old cocoyam plants with root rot symptoms (with chlorotic and necrotic old leaves) were collected. Two kg of surrounding soil were also collected per sampling point (two points) from a commercial plantation of cocoyam with root rot history in Nueva Guinea, South Caribbean Coast Autonomous Region, Nicaragua (11°42'39.3" N 84°26'46.2" O). The samples were processed in the Laboratorio de Micología at the Universidad Nacional Agraria [UNA] in Nicaragua. Pathogens associated to root rot were isolated, and their morphometric characteristics were recorded.

2.2. Isolation of root rot associated pathogens

Three methods in three cultivars (Table 1) were used to isolate the pathogens associated to root rot. Ten Petri dishes were used for each method and growth media.

Table 1. Composition of culture media used in the isolation of pathogens associated with root rot disease in cocoyam from soil serial dilutions, roots exhibiting symptoms of root rot, and cocoyam leaves as bait.

Growth medium	Ingredients	Concentration	Antibiotics	Concentration	Source
PDA[1]	Potato dextrose agar	39 g L^{-1}	---	---	Cañedo and Ames (2004)
AA+SE[2]	Agar	15 g L^{-1}	Streptomycin sulfate	400 mg L^{-1}	Nyochembeng et al. (2002); Olorunleke et al. (2014); Perneel et al. (2006); Tambong et al. (1999)
V8-PARB[3]	V8 Juice	100 mL L^{-1}	Pimaricin	400 µL L^{-1}	Gómez-Alpízar et al. (2011); Rodríguez Zamora and Ramírez Reynoza (2012); Steiner (1981)
	H_2O	900 mL L^{-1}	Ampicillin	250 mg L^{-1}	
	$CaCO_3$	1,75 g L^{-1}	Rifampicin	20 mg L^{-1}	
	Betasitosterol	0,05 g L^{-1}	Benomyl	20 mg L^{-1}	

[1] PDA: potato dextrose agar

[2] AA+SE: water agar + Streptomicine sulphate

[3] V8-PARB: V8 juice +Pimaricin, Ampicillin, Rifampicin and Benomyl

The isolation methods are described as follows:

- *Serial dilutions of the soil (DS).* 10 g of soil were taken from each of the collected soil samples, and they were diluted in 90 mL of sterile distilled water. The mix was stirred, and then the serial dilutions were done according to Germida and de Freitas (2008) and Martínez Francés et al. (2009). From the 10^{-3} and 10^{-4} samples, 200 µL were taken and placed in 5 Petri dishes containing different growth media (Table 1).
- *Root with root rot symptoms (RS).* Young roots of 0.5 cm-long and showing small necrotic stains were cut. They were washed with sterile distilled water and then with sodium hypochlorite ($NaCl_3$) at 1 % for 2 min. They were left in Petri dishes to grow (Table 1).
- *Soil samples with cocoyam leaves as bait [HQC].* The methodology of Almaraz-Sánchez et al. (2012) and Charlemagne and Xu (1997) was used with modifications. Pieces of 0.2 cm^2 healthy cocoyam leaves were sterilized and placed in an oven for 72 h at 60 °C for 15 min under UV light. A hundred g of soil sample was diluted in a beaker with 300 mL of sterile water. Roots and organic matter were removed, and the mix was left to rest for 10 min. The pieces of sterile leaves were incubated for 72 h at 28 °C (Perneel et al., 2006). The baits were removed, washed with sterile water, and dried in filter paper and placed in five Petri dishes for each growth medium (Table 1).

2.3. Identification, characterization and preparation of inoculants of isolated microorganisms

The identification and characterization of the isolated microorganisms that have been reported to be associated with root rot were carried out (Nzietchueng, 1984; Pacumbaba et al., 1992; Perneel et al., 2006, Saborío et al., 2004; Tambong et al., 1999).

2.3.1. *P. myriotylum*

The identification was done based on the morphological characteristics of the oomycete described by Ali-Shtayeh (1986), Plaats-Niterink (1981) and Waterhouse and Waterston (1966). The quantity and the diameter of the oogonia were considered, as well as the hyphal type and mycelial growth.

The isolates of *P. myriotylum* that presented greater mycelial growth, and more morphological characteristics were inoculated in the medium with higher growth rate (V8-PARB) and incubated at 28 °C for 5 days. After this period, the mycelium was mixed with sterile distilled water in a shaker for 2 min. The suspension was adjusted to concentration 1 x 10^3 oogonia mL^{-1} according to the methodology of Djeugap et al. (2016).

2.3.2. *Fusarium solani*

The growth of *Fusarium* was observed in three growth media, and the medium with higher fungal growth was selected for observation and description of morphometric characteristics and species identification, such as *F. solani*, according to the methodology of Seifert (1996) and Hafizi et al. (2013). The color and pigmentation of the colony were considered, as well as the growth rate and the morphology of the macroconidia and number of septa.

Before performing the inoculation of isolated *F. solani* in cocoyam plants, its pathogenicity was confirmed in 15-day-old melon plants after sowing in pots. The inoculum was prepared as a mother suspension, and 1 mL of this suspension was applied to melon seedlings. Two cm holes were made in the substrate next to the plant, in such a way that the roots were exposed, and 0.5 mL of the mother suspension was applied to each hole.

The inoculum was prepared from the Petri dishes with *F. solani* after seven days of growth in PDA medium. 20 mL of sterile distilled water was added and filtered through a sterile gauze into a beaker containing 75 mL of sterile distilled water, and the mixture was stirred using a vortex for 1 min. A drop of Tween 80 at 0.03% was added to the suspension for conidia counting, and serial dilutions of 10^{-1} and 10^{-2} were prepared. Fifty µL of the last dilution was taken to count the conidia with a hemocytometer under a microscope with 40X lens. The quantity of conidia was adjusted according to Corrales Ramírez et al. (2012) to a concentration of 1 x 10^4 spores mL^{-1}.

2.3.3. Isolation of bacteria from soil samples

A solution of 10 g of soil and 90 mL of sterile water was prepared, stirred and five serial dilutions were prepared. The 10^{-4} and 10^{-5} dilutions were used to take 0.1 mL of solution to place it in Petri dishes with tetrazolium medium [TZC]. The bacterial growth was observed for 24 h after the inoculation.

2.3.4. Bacteria Identification

Biochemical tests and stain protocols were performed to identify the bacterial genre:

- *KOH test:* two drops of KOH at 3% were added onto a slide with a small portion of the bacteria colony, then mixed for 2 min. If after the separation of the coverslip a viscous thread was formed, the test was positive, and the bacteria was considered Gram negative. However, if the thread was not formed, the reaction was negative, and the bacteria was considered Gram positive.
- *Oxidase test:* the methodology of Goszczynska et al. (2000) was used. Gram negative bacteria previously identified by the KOH test were used. Two drops of 1% tetramethyl-p-phenylenediamine dihydrochloride were added onto a piece of filter paper (1 cm^2). Using a sterile stick, a portion of young bacteria colony (24 h) was added. If after a minute the sample turned lilac, then it was positive for oxidase. However, if it did not change its color, then it was negative for oxidase.
- *Biochemical Tests:* to identify the species of bacteria, the oxidase-positive samples were used. Using a sterile stick, a small quantity of bacteria colony was taken and placed in test tubes with mannitol, sorbitol, dulcitol, lactose, maltose and cellobiose separately. Depending on the sugars in which bacteria tested positive and negative, the biovar was identified according to the description made by Schaad et al. (2001). After the identification, the bacteria were grown through striation technique on the solid media of nutritious agar [AN] to purify the colonies.

The solution preparation for flowerpot-soil inoculation was done according to the method used by Rodríguez Zamora and Ramírez Reynoza (2012). The bacteria were diluted in sterile distilled water and adjusted to 6 x 10^8 UFC mL^{-1}.

2.4. Pathogenicity tests did on the greenhouse

The pathogenicity tests were done on a greenhouse following the indications:

2.4.1. Location of the study for the pathogenicity tests

The assay was established in the greenhouse of the Centro Nacional de Investigación Agropecuaria of the Instituto Nicaragüense de Tecnología Agropecuaria [CNIA-INTA], Managua, located on the coordinates 12°07'55.3"N 86°08'49.1"O. An average temperature between 31.5 and 35.83 °C, maximum between 335 and 39.3 °C, and minimum between 28 and 32.3 °C, was recorded during the 11 weeks that the assay lasted (Figure 1).

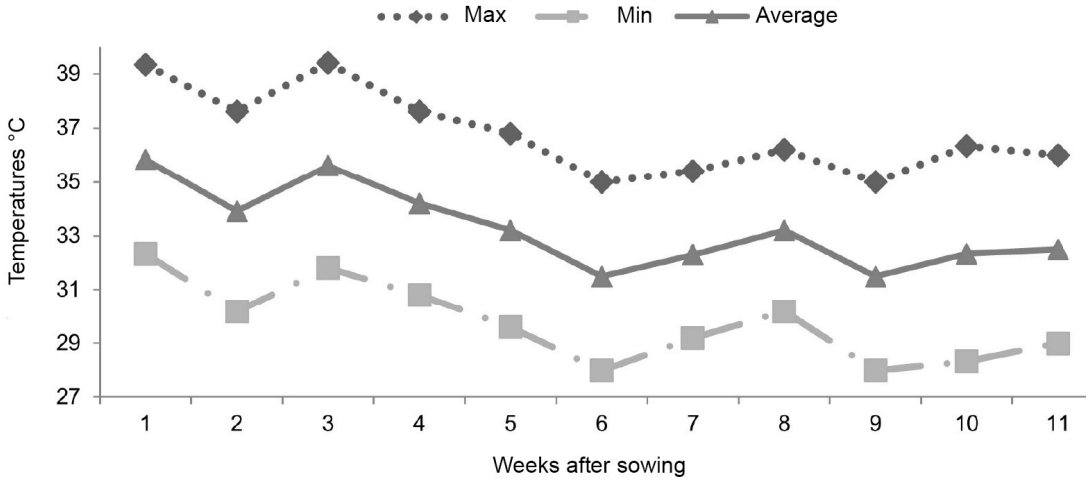

Figure 1. Average, minimum and maximum temperature recorded in the greenhouse at Centro Nacional de Investigación Agropecuaria of the Instituto Nicaragüense de Tecnología Agropecuaria.

2.4.2. Application of Koch's postulates for the pathogenicity tests

In vitro plants of lilac cocoyam were sown in flowerpots with soil collected from a site with previous cocoyam commercial plantations, in the Nueva Guinea municipality. This area was selected for having among the highest production of cocoyam in Nicaragua and soils with Ultisoles-HC Udults classification (soils in senile state or at the last stage of degradation by chemical properties caused by red or yellow rain) according to Instituto Nicaragüense de Estudios Territoriales [INETER] and Universidad Nacional Agraria [UNA] (2015). The soil was sterilized in an oven at 240 °C for 72 h and mixed with inorganic substrate (Kekkila) in a 3:1 proportion. Two kg of this material was used per flowerpot.

2.4.3. Used treatments

Fifteen vitroplants of 15 cm height with 100 days of acclimatization in greenhouse and with at least three leaves for treatments were used. To evaluate the effects, three plants per treatment were assessed on the 7th, 21st, and 35th days after inoculation [ddi].

Eight treatments corresponding to the three isolated pathogens from previous identification and characterization, their combination and a control group (without pathogen application) were evaluated:

- T.1: *Pythium myriotylum* [P].
- T.2: *Fusarium solani* [F].
- T.3: *Ralstonia solanacearum* [R].

- T.4: *Pythium myriotylum* + *Fusarium solani* [P+F].
- T.5: *Pythium myriotylum* + *Ralstonia solanacearum* [P+R].
- T.6: *Fusarium solani* + *Ralstonia solanacearum* [F+R].
- T.7: *Pythium myriotylum* + *Fusarium solani* + *Ralstonia solanacearum* [P+F+R].
- T.8: Control group (without pathogen inoculation) [T].

P. myriotylum was inoculated with 18.5 mL of inoculum suspension per flowerpot (Djeugap et al., 2016). To inoculate *F. solani*, 1 mL (1 x 10^4 spores mL^{-1}) of the suspension was used in each flowerpot (Corrales Ramírez et al., 2012); and it was inoculated according to the methodology described by Rodríguez Zamora and Ramírez Reynoza (2012). Three mL of the bacterial solution was applied in each flowerpot.

2.4.4. Re-isolation of cocoyam's pathogens

The plants showing symptoms of root rot were processed in the lab at the 35 ddi to re-isolate the pathogen present in the roots and substrate. The culture media PDA, AA + SE and V8-PARB were used, and the morphological characteristics were evaluated to identify the presence of the inoculated pathogen.

2.4.5. Evaluated variables

Root variables were recorded to evaluate the symptoms of root rot in the plants. The variables are described as follows:

a) *Healthy root number.* The number of roots with no symptoms or lesions that could indicate root rot symptomatology.
b) *Number of infected roots*. The number of all the plant roots with visible necrosis, rot or barking.
c) *Length of roots*. The roots were measured from the beginning of the plant's corm until the tip, the measurements were taken from 5 roots randomly taken.
d) *Disease incidence in roots.* It was evaluated with the equation [1] (Djeugap et al. 2016)

$$I = \frac{Number\ of\ infected\ roots}{Total\ number\ of\ roots} x\ 100 \quad [1]$$

2.5. Data Analysis

A variance analysis [ANOVA] and separation of media of Fisher $\alpha = 0.05$ were used to analyse the variables of healthy roots, number of affected roots, and length of roots. The program InfoStat version 2018e was used to determine the statistical differences among treatments.

3. Results

3.1. Isolation of associated pathogens to root rot according to the methodology

A 119 isolates of *Pythium* spp. and 61 isolates of *Fusarium* spp. were registered. 16 isolates of *Fusarium* spp. and seven of *Pythium* spp were obtained with the method HQC in PDA media. Sixteen isolates of *Fusarium* spp. and 30 of *Pythium* spp. were obtained in the media AA+SE, and 33 isolates of *Pythium* spp. were obtained from the medium V8-PARB (Tabla 2).

Using the RS method, growth of *Fusarium spp.* was obtained on PDA medium. With the DS method, 27 isolates of *Fusarium spp.* and 21 isolates of *Pythium spp.* grew on PDA medium, while on AA+SE medium, one isolate of *Fusarium spp*. and 28 isolates of *Pythium spp*. grew. No growth was observed on V8-PARB medium (Table 2).

Fusarium spp. grew on PDA medium and presented the characteristic septate mycelial growth with pink and lilac colonies, macroconidia with curvatures between 3-4 septa, and it was identified after seven days of inoculation. The growth of *Fusarium* in AA+SE was scarce, and it was not possible to distinguish reproductive structures, which made the identification of species challenging in this medium.

Table 2. Number of isolates (*Fusarium* spp. and *Pythium* spp.) obtained with the isolation methods: leaves as bait (*Xanthosoma violaceum*) [HQC], roots with symptoms [RS] and serial dilutions of soil [DS], in the culture media Potato Dextrose Agar [PDA], water agar with streptomycin sulfate [AA+SE] and V8 Juice with Pimaricin, Ampicillin, Rifampicin and Benomil [V8-PARB].

Growth Medium	HQC		RS		DS		Total	
	Fusarium spp.	*Pythium* spp.	*Fusarium* spp.	*Pythium* spp.	*Fusarium* spp.	*Pythium* spp.	*Fusarium* spp.	*Pythium* spp.
PDA	16	7	1	0	27	21	44	28
AA+SE	16	30	0	0	1	28	17	58
V8PARB	0	33	0	0	0	0	0	33
Total	32	70	1	0	28	49	61	119

In the culture medium AA+SE, 58 isolates of *Pythium* spp. were obtained with a lateral and scarce mycelial growth within the growth medium. Using the HQC method, 70 isolates of *Pythium* spp. were obtained, where 58.8% had fluffy mycelial growth. The mycelial growth was ascending and abundant in the Petri dish in the V8-PARB culture medium (33 isolates) compared to the PDA (7 isolates) and AA+SE (30 isolates) culture media and was the only pathogen that grew in this medium.

In the AA+SE culture medium, 58 isolates of *Pythium* spp. were obtained, exhibiting lateral and sparse mycelial growth within the medium. Using the HQC method, 70 isolates of *Pythium* spp. were obtained, representing 58.8% of the total *Pythium* spp. isolates, with highly cottony, ascending, and abundant mycelial growth in Petri dishes on the V8-PARB culture medium (33 isolates) compared to the PDA (7 isolates) and AA+SE (30 isolates) culture media. *Pythium* spp. was the only pathogen that grew in the V8-PARB medium.

3.2. Pathogen identification

The pathogen *Pythium myriotylum* was identified three days after being isolated by the HQC method and cultured on V8-PARB medium at 28 °C. It showed white, ascendant and cottony mycelium with a great quantity of circular oogonia of 23 µm in diameter with double wall and 1 to 3 antheridia per oogonia, with abundant oospores with coenocytic hypha and development of filamentous sporangia (Figure 2). All the virulent isolates of *P. myriotylum* in cocoyam grow better at 28 °C (Perneel et al., 2006). The species of *P. myriotylum* could not be identified in other media due to the lack of reproductive structures and low mycelium development.

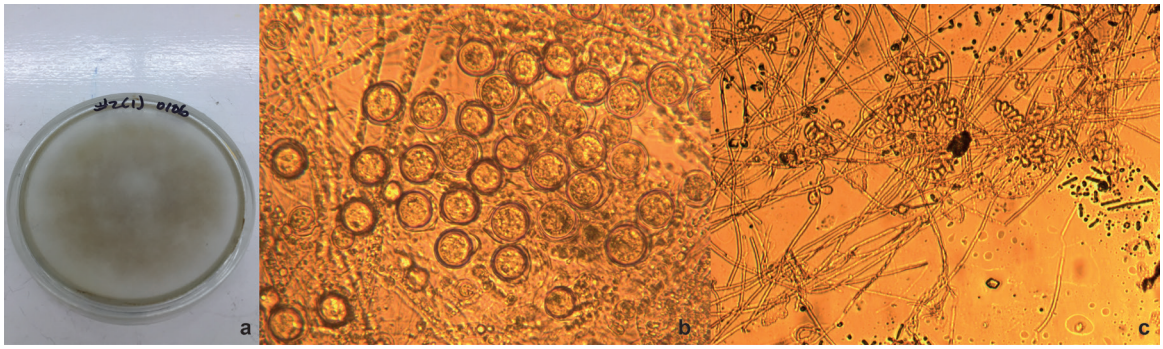

Figure 2. *Pythium myriotylum* structures observed under the microscope 40X and identified by taxonomic keys a) Growth of *Pythium myriotylum* in culture medium V8 with Pimaricin, Ampicilin, Rifampicin and Benomil medium b) Oogonia c) Coenocytic hyphae and sporangia.

Fusarium solani was isolated and identified with the DS method in PDA medium. It presented aerial mycelium and white-grey-pink cottony texture with colorful pigmentation (lilac, purple, brown), septate hyphae and abundant microconidia with 3-4 septa, similar to the one described by Seifert (1996), Rodríguez Zamora and Ramírez Reynoza (2012), and Hafizi et al. (2013) (Figure 3). The species could not be identified in the PDA isolates from the RS and HQC methods due to the presence of small quantity of mycelium (Table 2).

Ralstonia solanacearum biovar 2 was identified after the KOH, oxidase and biochemical tests. It tested positive for sugars: maltose, cellobiose and lactose, which belongs to the bacteria *R. solanacearum* according to the description of Schaad et al. (2001).

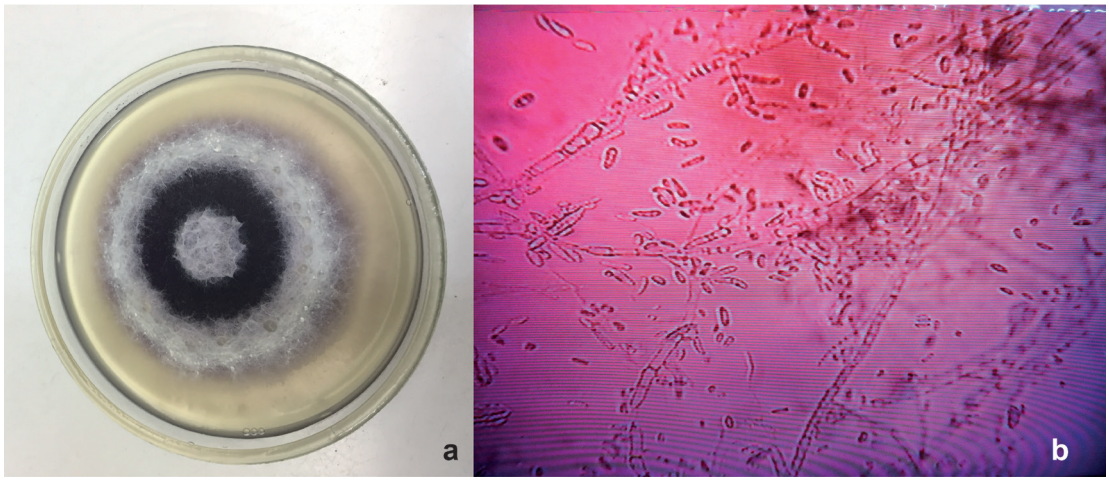

Figure 3. a) Growth of *F. solani* in PDA culture medium eight days after inoculation in medium, b) Morphological identification of *F. solani*, hyphae and macroconidia.

3.3. Pathogenicity Tests based on Koch's postulates

In the pathogenicity tests, the combined treatments with *P. myriotylum* showed symptoms in the roots. All the variables are listed:

- The evaluated plants shown average values of healthy roots in ranges of 4.7-20.7 with 5.7-24.8 cm in length and an average range of 0-11 diseased roots. Significant differences were recorded between treatments in all variables and evaluations with exception of the root length at 35 ddi (Table 3).
- The number of diseased roots in the R, F, F+R and T treatments had the same category in all evaluations, but plants with roots without symptoms were also observed (Table 3).
- The P+R, P, P+F+R and P+F treatments were placed in the second category, with necrotic roots, rottenness, lesions, and barking, typical symptoms of root rot. These lesions are attributed to a comon pathogen in these treatments: *P. myriotylum* (Table 3):

The plants of the treatments F, R, F+R and T had normal growth and the percentage of disease incidence in the roots was less than 10% in all the evaluations. On the other hand, the roots in the P, P+F, P+R and P+F+R treatments showed an increase in disease incidence after 7 days of being inoculated, with necrosis symptoms and root rot (Figure 4).

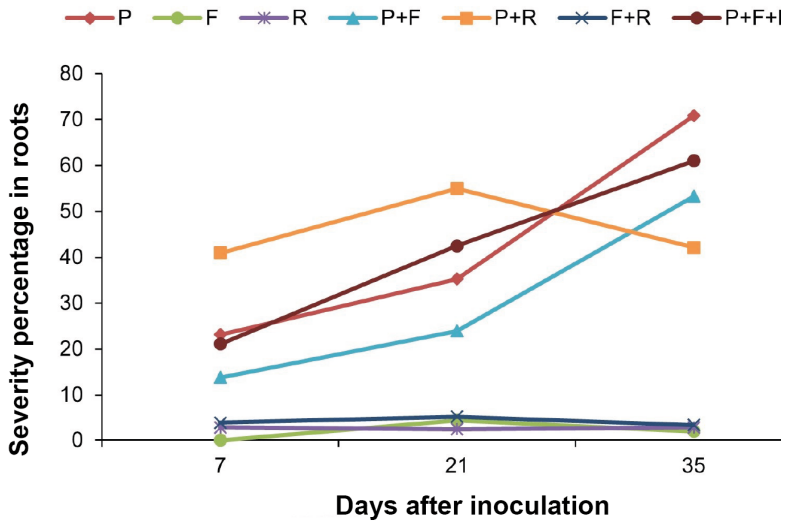

Figure 4. Percentage of disease incidence registered in the roots of in vitro plants of the cultivar quequisque Lila (*Xanthosoma violaceum*), inoculated with pathogenic isolates *P. myriotylum* [P], F. solani [F], R. solanacearum [R] applied alone and in combination (P + F, P + R, F + R, P + F + R) and control [T] without pathogen inoculation.

Table 3. Fisher's mean separation (α = 0,05), P value, coefficient of variation [CV] and coefficient of determination [R^2] of the number of healthy roots, affected roots and root length of vitroplants of the Lila cocoyam (*Xanthosoma violaceum*) cultivar evaluated at 7, 21 and 35 days after inoculation, in pathogenicity tests based on Koch's postulates.

Treatments	Number of affected roots			Number of healthy roots			Root length (cm)		
	Days after inoculation								
	7	21	35	7	21	35	7	21	35
R	0,33 a	0,33 a	0,33 a	13,00 a	12,00 b c	1 3 , 0 0 abc	8,78 cd	22,63 ab	19,33 a
F	0,00 a	0,67 a	0,33 a	14,67 a	14,33 abc	18,00 a	9,71 bcd	12,70 cd	16,97 a
F+R	0,67 a	0,67 a	0,67 a	16,33 a	11,00 bc	14,33 ab	5,68 d	24,78 a	19,16 a
T	0,67 a	0,33 a	0,67 a	13,00 a	16,67 ab	15,33 ab	12,27 bc	15,59 bcd	17,28 a
P+R	6,67 b	6,67 b	9,00b	9,67 a	11,00 bc	13,67 ab	20,20 a	13,00 cd	22,77 a
P	4,67 b	8,67 b	10,67 b	15,33 a	16,33 bc	4,67 d	9,40 bcd	11,44 d	19,17 a
P+F+R	4,67 b	6,37 b	11,00 b	17,00 a	8,33 c	7,33 cd	13,83 b	20,27 abc	17,15 a
P+F	2,67 b	6,00 b	11,00 b	17,33 a	20,67 a	9,67 bcd	6,72 d	11,30 d	12,67 a
P valor	0,0001	0,0001	0,0001	0,1086	0,0174	0,0028	0,0002	0,0112	0,5331
CV (%)	44,72	54,21	23,95	21,29	26,6	27,8	25,53	28,43	29,16
R^2	0,87	0,80	0,96	0,47	0,61	0,70	0,79	0,63	0,28

3.4. Re-isolation of cocoyam plant pathogens with root rot symptoms

The isolated pathogens from plants with root rot symptoms were the same pathogens used for inoculation in the pathogenicity tests, fulfilling with the 4th Koch's postulate according to Agrios (2005) (Table 4). *Pythium myriotylum* was isolated from all the treatments (P, P+F, P+R and P+F+R) in the growth media AA+-SE and V8+PARB (Table 4).

Table 4. Re-isolation of pathogens found in cocoyam (*Xanthosoma violaceum*) plants with symptoms of root rot dissease.

Growth medium	P	P+F	P+R	P+F+R
PDA[1]	---	*F. solani*	---	*F. solani*
AA + SE[2]	*P. myriotylum*	*P. myriotylum*	*P. myriotylum*	*P. myriotylum*
V8 + PARB[3]	*P. myriotylum*	*P. myriotylum*	*P. myriotylum*	*P. myriotylum*
TZC[4]	---	---	*R. solanacearum*	*R. solanacearum*

[1] PDA: potato dextrosa agar
[2] AA+SE: water agar+ Streptomicine sulphate
[3] V8+PARB: V8 juice + Pimaricin, Ampiciline, Rifampicine and Benomil
[4] TZC: Tetrazolium

4. Discussion

Using the method HQC in combination with the growth medium V8+PARB was the most effective methods to isolate and grow *Pythium myriotylum.* Growing in aqueous medium and being in contact with leaves facilitated the germination and development of *P. myriotylum,* which facilitated its isolation due to the increased quantity of mycelia on the leaf pieces. Soil humidity can affect the production of sporangia, development of antheridia, oogonia and development of mycelium of *Pythium* (Yudiarti et al., 2006). These combination of techniques guarantee to have isolates of *P. myriotylum* without the need of using cocoyam plants with recent root rot symptoms. Sometimes, deteriorated tissue containing dormant *Pythium* propagules can be used for isolation. Therefore, it is preferable that the medium allows the spores to germinate and develop more easily (Jeffers & Martin, 1986).

Other authors isolated *Pythium* with sweet pepper (*Capsicum annuum* L.) bait tissue, in saturated moist soil and cornmeal agar and V8 juice culture media supplemented with the antibiotics pimaricin, vancomycin, and pentachloronitrobenzene. They achieved abundant mycelium growth that saturated the Petri dish in the growth medium V8, which facilitated the identification and manipulation of *Pythium* (Urrutia-Anaya & Pacheco-Aguilar, 2009). The same growth was observed during this study in the V8+PARB medium, which facilitated the identification of *P. myriotylum* in comparison to the other selected media (AA+SE and PDA), where the growth was lower and it did not have reproductive structures to carry out the identification of the species. Therefore, it was only possible to identify until genre level for the isolates found in these media.

Having a culture medium where *P. myriotylum* can be grown is important to facilitate its identification. The medium of V8 juice with different antibiotics was previously used, and the importance in the use of antibiotics in selective growth media for the development of pathogens was shown (Jeffers & Martin, 1986; Rodríguez Zamora & Ramírez Reynoza, 2012; Urrutia-Anaya & Pacheco-Aguilar, 2009). The use of antibiotics (pimaricin, ampicillin, and rifampicin) and fungicides (benomyl) in the V8+PARB growth medium inhibited the growth of bacteria and fungi present in the soil, which allowed an exuberant development of *P. myriotylum*, being able to observe reproductive structures that facilitated its identification. Other the fungi and bacteria growth in the AA+SE and PDA media, sometimes they saturated the media, and it was not possible to identify the species present in the media. Although the profusely production of structures in the soil, isolation of *Pythium* spp. is challenging because *Pythium* grows less than other fungi or bacteria. Thus, a specific technique is required to recover it (Yudiarti et al., 2006).

The isolates of *P. myriotylum* that affect cocoyam are different of the typical isolates of *P. myriotylum* that cause damping off in other host plants and could be easily identified from other isolates of *P. myriotylum* due to its optimal growing temperature in the lab (28 °C) (Perneel et al., 2006), and used during this study to isolate and multiply the oomycete. For the isolation and identification of *Pythium,* media that stimulate the development of sporangia, oogonia and antheridia is necessary for its identification because the morphology of the sporangia in *Pythium* is the key for its identification (Urrutia-Anaya & Pacheco-Aguilar, 2009).

The challenges in the isolation of *P. myriotylum* from roots (visibility of the first symptoms in roots and specific growth media) could explain the differences in the identification of the causal agent of root rot in cocoyam. Some authors attribute the root rot symptoms to *F: solani*, *Sclerotium rolsii* Sacc and a complex of fungi (Dávila Martínez, 2011; Dávila Martínez et al., 2016; Folgueras Montiel et al., 2015; Saborío et al., 2004). In the variables for disease incident in roots and number of diseased roots (Table 3 and Figure 3), *P. myriotylum* is present in the treatments that show plants with root rot symptoms: necrosis, and rottenness in roots. These support the evidence the results from Nzietchueng (1984); Pacumbaba et al. (1992); Tambong et al. (1999) and Rodríguez Zamora & Ramírez Reynoza (2012) in Nicaragua, who reported *P. myriotylum* as the only causal agent of root rot in cocoyam.

5. Conclusions

The isolation method for *Pythium myriotylum,* using the soil with cocoyam leaves as bait, was effective. Higher quantity of isolates of *P. myriotylum* with abundant mycelium were obtained combining this method with the growth medium V8 juice plus pimaricin, ampicillin, rifampicin, and benomyl. This facilitated its morphometric identification. *P. myriotylum* triggered typical symptoms of root rot in the lilac cocoyam vitroplants. The inoculation with *F. solani* and *R. solanacearum,* individually or mixed, did not present root rot symptoms.

Contributor roles

- Heeidy Guadalupe Corea Narváez: conceptualization, formal analysis, investigation, methodology, writing – original draft.
- Rayan Oniel Gonzalez Moya: investigation, methodology.
- Guillermo del Carmen Reyes Castro: conceptualization, funding acquisition, project administration, resources, supervision.

Ethical Implications

Ethics approval not applicable.

Conflicts of Interest

The authors declare that they have no affiliation with any organization with a direct or indirect financial interest that could have appeared to influence the work reported.

References

Acebedo Rivera, J. A., & Navarro Matute, E. R. (2010). *Efectos del mal seco (Phytium myriotylum Drechs) en campo y sombreadero sobre la agromorfología de 15 accesiones de quequisque (Xanthosoma spp.), desarrollo de síntomas y detección microbiológica*. Universidad Nacional Agraria. https://repositorio.una.edu.ni/id/eprint/2120

Agrios, G. N. (2005). *Fitopatología* (2ª ed.). Limusa S.A.

Ali-Shtayeh, M. S. (1986). *The genus pythium in the west bank and Gaza Strip.* Research and Documentation Centre, An-Najah National University. https://staff-old.najah.edu/msshtayeh/published-book/genus-pythium-west-bank-and-gaza-strip

Almaraz-Sánchez, A., Alvarado-Rosales, D., & Saavedra-Romero, L. de L. (2012). Trampeo de *Phytophthora cinnamomi* en bosque de encino con dos especies ornamentales e inducción de su esporulación. *Revista Chapingo Serie Ciencias Forestales y del Ambiente, 19*(1), 5-12. www.doi.org/10.5154/r.rchscfa.2011.09.062

Bejarano-Mendoza, C. A., Zapata, M., Bosques, A., Rivera-Amador, E., & Liu, L. J. (1998). *Sclerotium rolfsii* como componente del complejo patológico causante del mal seco de la yautía (*Xanthosoma sagittifolium*). *The Journal of Agriculture of the University of Puerto Rico*, *82*(1-2), 85-95. https://doi.org/10.46429/jaupr.v82i1-2.3822

Cañedo, V., & Ames, T. (2004). *Manual de laboratorio para el manejo de hongos entomopatógenos*. Centro Internacional de la Papa. https://cipotato.org/wp-content/uploads/2014/09/AN65216.pdf

Charlemagne, T. T. J., & Xu, T. (1997). *Pythium* species from cocoyam farm soils in Cameroon. *Fungal Science, 12*(1/2), 9-15.

Corrales Ramírez, L. C., Sánchez Leal, L. C., Cuervo Andrade, J. L., Joya, J. A., & Márquez, K. (2012). Efecto biocontrolador de *Bacillus* spp., frente a *Fusarium* sp., bajo condiciones de invernadero en plantas de tomillo (*Thymus vulgaris* l.). *Revista Nova Publicación Científica en Ciencias Biomédicas*, *10*(17), 64-82. https://doi.org/10.22490/24629448.518

Dávila Martínez, A. (2011). *Las pudriciones secas de la malanga (Xanthosoma y Colocasia). Etiología y sintomatología*. Universidad Central Marta Abreu de Las Villas. https://dspace.uclv.edu.cu/handle/123456789/2173

Dávila Martínez, A., Herrera Isla, L., Folgueras Montiel, M., & Espinosa-Cuellar, E. (2016). Patogenicidad de especies fúngicas presentes en los rizomas de malanga (*Xanthosoma* y *Colocasia*). *Centro Agrícola*, *43*(2), 49-58. http://cagricola.uclv.edu.cu/descargas/pdf/V43-Numero_2/cag07216.pdf

Djeugap, F. J., Azia, A. T., Tita, N. C., Eko, D., & Fontem, D. A. (2016). Influence of compost types and fungicide application on plant growth and suppressiveness of cocoyam [*Xanthosoma sagittifolium* (L.) Schott] root rot disease. *International Journal of Agriculture and Bioscience*, *5*(1), 32-37. https://www.ijagbio.com/pdf-files/volume-5-no-1-2016/32-37.pdf

Folgueras Montiel, M., Herrera Isla, L., & Rodríguez Morales, S. (2015). El mal seco de la malanga (*Xanthosoma* y *Colocasia*) en Cuba. *Revista Agricultura Tropical*, *1*(1), 62-65.

Germida, J. J., & de Freitas, J. R. (2008). Cultural methods for soil and root associated microorganisms. En M. R. Carter, y E. G. Gregorich (eds.), *Soil sampling and methods of analysis* (2nd ed.). CRC Press. https://doi.org/10.1201/9781420005271

Giacometti, D. C., & Leon, J. (1994). Tannia, yautia (*Xanthosoma sagittifolium*). En J. E. Hernández Bermejo, y J. León (eds.), *Neglected Crops: 1492 from a different perspective* (pp. 253-258). FAO Plant Production

and Protection Series No.26. Food and Agriculture Organization of the United Nations. https://www.fao.org/4/t0646e/t0646e.pdf

Gómez-Alpízar, L., Saalau, E., Picado, I., Tambong, J. T., & Saborío, F. (2011). A PCR-RFLP assay for identification and detection of *Pythium myriotylum*, causal agent of the cocoyam root rot disease. *Letters in Applied Microbiology*, *52*(3), 185-192. https://doi.org/10.1111/j.1472-765X.2010.02998.x

Goszczynska, T., Serfontein, J. J., & Serfontein, S. (comps.). (2000). *Introduction to practical phytobacteriology: A manual for phytobacteriology* (1st ed.). Safrinet-Loop of bioNet-International. https://ipmil.cired.vt.edu/wp-content/uploads/2014/06/Practical-Bacteriology-Guide-copy.pdf

Hafizi, R., Salleh, B., & Latiffah, Z. (2013). Morphological and molecular characterization of *Fusarium solani* and *F. oxysporum* associated with crown disease of oil palm. *Brazilian Journal of Microbiology*, *44*(3), 959-968. https://doi.org/10.1590/S1517-83822013000300047

Instituto Nicaragüense de Estudios Territoriales [INETER], & Universidad Nacional Agraria [UNA]. (2015). *Mapa de suelos de la República de Nicaragua*. INETER.

Jeffers, S. N., & Martin, S. B. (1986). Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease*, *70*(11), 1038-1043. https://doi.org/10.1094/PD-70-1038

Martínez Francés, M. Á., Lacasa Plasencia, A., & Tello Marquina, J. C. (2009). *Ecología de la microbiota fúngica de los suelos de los invernaderos de pimiento y su interés agronómico*. Ministerio de Medio Ambiente y Medio Rural y Marino. https://www.mapa.gob.es/es/agricultura/publicaciones/ECOLOGIA%20MICROBIOTA%20baja_tcm30-57876.pdf

Nyochembeng, L. M., Pacumbaba, R. P., & Beyl, C. A. (2002). Calcium enhanced zoospore production of *Pythium myriotylum in vitro*. *Journal of Phytopathology*, *150*(7), 396-398. https://doi.org/10.1046/j.1439-0434.2002.00759.x

Nzietchueng, S. (1984). Root rot *Xanthosoma sagittifolium* caused by *Pythium myriotylum* in Camerun. In E. R. Terry, E. V. Doku, O. B. Arene, & N. M. Mahungu (eds.), *Tropical root crops: production and uses in Africa. Proceedings of the second triennial Symposium of the international society of tropical Root crops* (pp. 185-188). International Development Research Centre. https://idl-bnc-idrc.dspacedirect.org/server/api/core/bitstreams/5598eb2e-0462-4abe-a928-38e051112bbb/content

Olorunleke, F., Adiobo, A., Onyeka, J. T., & Höfte, M. (2014). Cocoyam root rot disease caused by *Pythium myriotylum* in Nigeria. In *Tropentag 2014: Conference on International Research -on Food Security, Natural Resource Management and Rural Development*. Prague, Czech Republic. https://www.tropentag.de/2014/abstracts/links/Houmlfte_5jvOBYj9.pdf

Pacumbaba, R. P., Wutoh, J. G., Eyango, S. A., Tambong, J. T., & Nyochembeng, L. M. (1992). Isolation and pathogenicity of rhizosphere fungi of cocoyam in relation to cocoyam root rot disease. *Journal of Phytopathology*, *135*(4), 265-273. https://doi.org/10.1111/j.1439-0434.1992.tb04312.x

Perneel, M., Tambong, J. T., Adiobo, A., Floren, C., Saborío, F., Lévesque, A., & Höfte, M. (2006). Intraspecific variability of *Pythium myriotylum* isolated from cocoyam and other host crops. *Mycological Research*, *110*(5), 583-593. https://doi.org/10.1016/j.mycres.2005.12.002

Plaats-Niterink, J. van der. (1981). Monograph of the genus *Pythium*. *Studies in Mycology,* (21). https://www.studiesinmycology.org/sim/Sim21/full%20text.htm

Reyes Castro, G. (2006). Studies on cocoyam (*Xanthosoma* spp.) in Nicaragua, with emphasis on *Dasheen mosaic virus.* Swedish University of Agricultural Sciences. https://res.slu.se/id/publ/13224

Reyes Castro, G. del C., Corea Narváez, H. G., & Guatemala Ortega, T. (2013). *Guía del manejo agronómico del quequisque en Nicaragua*. Programa Propemce. https://cenida.una.edu.ni/relectronicos/RENF01R457g.pdf

Rodríguez Zamora, M. J., & Ramírez Reynoza, D. J. (2012). *Diagnóstico de los agentes causales del mal seco en el cultivo de quequisque (Xanthosoma* spp*.) en el municipio de Nueva Guinea, Nicaragua*. https://repositorio.una.edu.ni/2161/

Saavedra, D., & Reyes, G. (2012). *Prospección tecnológica para el manejo de mal seco en quequisque*. FUNICA.

Saborío, F., Umaña, G., Solano, W., Amador, P., Munos, G., Valerin, A. T., Torrez, S., & Valverde, R. (2004). Induction of genetic variation in *Xanthosoma* spp. In International Atomic Energy Agency, *Genetic improvement of under-utilized and neglected crops in low income food deficit countries through irradiation and related techniques* (pp. 143-154). International Atomic Energy Agency. https://www.iaea.org/publications/7155/genetic-improvement-of-under-utilized-and-neglected-crops-in-low-income-food-de-

ficit-countries-through-irradiation-and-related-techniques
Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for identification of plant pathogenic bacteria* (3rd ed.). American Phytopathological Society Press. https://doi.org/10.1046/j.1365-3059.2001.00635.x
Seifert, K. (1996). *Fuskey: Fusarium interactive key, taxonomic information systems*. Agriculture & Agri-Food Canada. https://www.pilzforum.eu/attachment/433750-fusarium-key-pdf/
Sinobas, J., Varés, J., & Rodríguez, E. (1999). Influencia del tipo de «cebo» y la temperatura en el aislamiento y desarrollo de *Pythium* spp. *Boletín de Sanidad Vegetal, Plagas, 25*(2), 131-142. https://www.mapa.gob.es/app/publicaciones/art_datos_art.asp?articuloid=836&codrevista=Plagas
Steiner, K. G. (1981). A root rot of macabo (*Xanthosoma* sp.) in Cameroun, associated with *Pythium myriotylum*. *Journal of Plant Diseases and Protection*, *88*(10), 608-613. https://www.jstor.org/stable/43216229
Tambong, J. T., Poppe, J., & Höfte, M. (1999). Pathogenicity, electrophoretic characterization and in planta detection of the cocoyam root rot disease pathogen, *Pythium myriotylum*. *European Journal of Plant Pathology*, *105*, 597-607. https://doi.org/10.1023/A:1008745732733
Waterhouse, G. M., & Waterston, J. M. (1966). *Pythium myriotylum* . [Descriptions of Fungi and Bacteria]. *Descriptions of Fungi and Bacteria*. https://doi.org/10.1079/DFB/20056400118
Urrutia-Anaya, I., & Pacheco-Aguilar, J. R. (2009). *Aislamiento e identificación de Pythium* sp., *un fitopatógeno de interés agrícola en el estado de Querétaro*. Universidad Autónoma de Querétaro. https://www.uaq.mx/investigacion/difusion/veranos/memorias-2009/11VCRC_46/35_Urrutia_Anaya_Color.pdf
Yudiarti, T., Jensen, D. F., & Hockenhull, J. (2006). Isolation and identification of *Pythium* from soil. *Jurnal Pengembangan Peternakan Tropis*, *3*(31), 141-146. http://eprints.undip.ac.id/6894/