Screening for resistance to Yam Anthracnose Disease

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Objectives

This field/lab guide is to assist you in screening landraces, elite clones, segregating populations and breeders lines of yam for resistance to Yam anthracnose disease (YAD) through:

- Isolation of pathogen, preparation of inoculum, inoculation techniques
- Rapid screening techniques: Detached leaf assay
- Utilization of digital phenotyping _Leaf Doctor or Assess 2.0
- Scale of scoring for the disease

Materials

- Healthy and infected yam plants
- Laboratory equipment for screening
- Field equipment for screening
- Identification guide (pictorial)

Protocols

- Identification of diseased plant on the field
- Isolation techniques
- Inoculation methods (detached leaf and whole plants)
- Laboratory identification of isolates
- Detached leaf assay
- Use of digital imaging for scoring

Introduction

Yam Anthracnose Disease (YAD) can cause up to 80% yield loss where it is endemic. Due to the losses incurred through the effect of diseases such as YAD, yam breeders and other affiliated scientists are trying to breed for plant materials with resistance to this disease. Resistance breeding is more acceptable in these days of climate change because they are more environment- friendly and cost effective. In order to be more effective in the breeding process, there is a need to be able to identify accurately and quickly, that is, early in the breeding cycle, plants with resistance to the diseases. Considering the importance of yams as an income generating staple, and its socio-cultural significance especially in West Africa, there is a need for the distribution of disease free yam propagules. This can be enhanced by being able to screen for the presence or absence of the disease. To do this effect, we need to be able to identify the pathogen accurately, inoculate and take accurate readings of genotypic reaction to the disease in order to be able to proffer a lasting solution for its control.

Anthracnose disease

Anthracnose is a fungal disease caused by many closely related fungi in the genus *Colletotrichum*, the pathogen responsible for the yam anthracnose disease is *Colletotrichum gloeosporioides*. It attacks mostly *D. alata* in the yam family; even though it can also attack other yam spps. The disease can affect the leaves, vines and tubers.

Types of *Colletotrichum gloeosporioides* (Cg)

According to the studies by Abang *et al.,* (2002), there are four types of *Colletotrichum gloeosporioides* in Nigeria: Slow growing grey (SGG), Fast growing grey (FGG), Fast growing salmon (FSG) and Slow growing Olive (SGO).

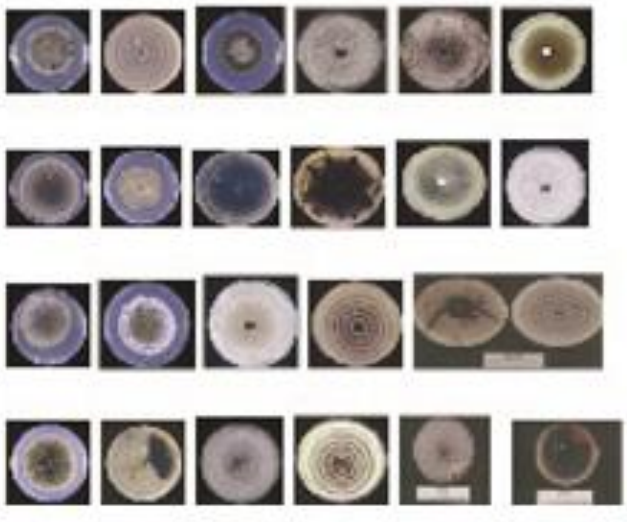

Figure 1. Typical growth patterns of *Colletotrichum gloeosporioides* (Ayodele *et al.*, (2004)

Environmental condition favoring the development

Environment is a very important part of the disease triangle. In the case of YAD, moisture is required for the development and germination of the fungal spores as well as for infection of the plant. A humid and rainy environment enhances the growth and spread of the disease on the field.

Mode of transmission of YAD and control

It can be spread by wind, rain, insects and garden tools. It is mostly spread through rain splashes of the soil containing spores on the plants. The use of resistant variety is the best control, however fungicides can be applied and clearing and ploughing in of left over debris is encouraged as a cultural control because the pathogens can overwinter (remain dormant until a favourable condition starts) in the plant debris.

Description and field symptoms:

Symptoms include spots or blight. Severe infection results in vine dieback, defoliation and tuber dry rot. About 7 types of spots and 4 types of blights have been identified as its symptoms on yam leaves (Ayodele *et al.,* 2004). It presents as a dark lesion which is usually surrounded with a yellow halo. They can also be seen as dark brown rings surrounding a light brownish necrotic portion. (Figures 2 and 3)

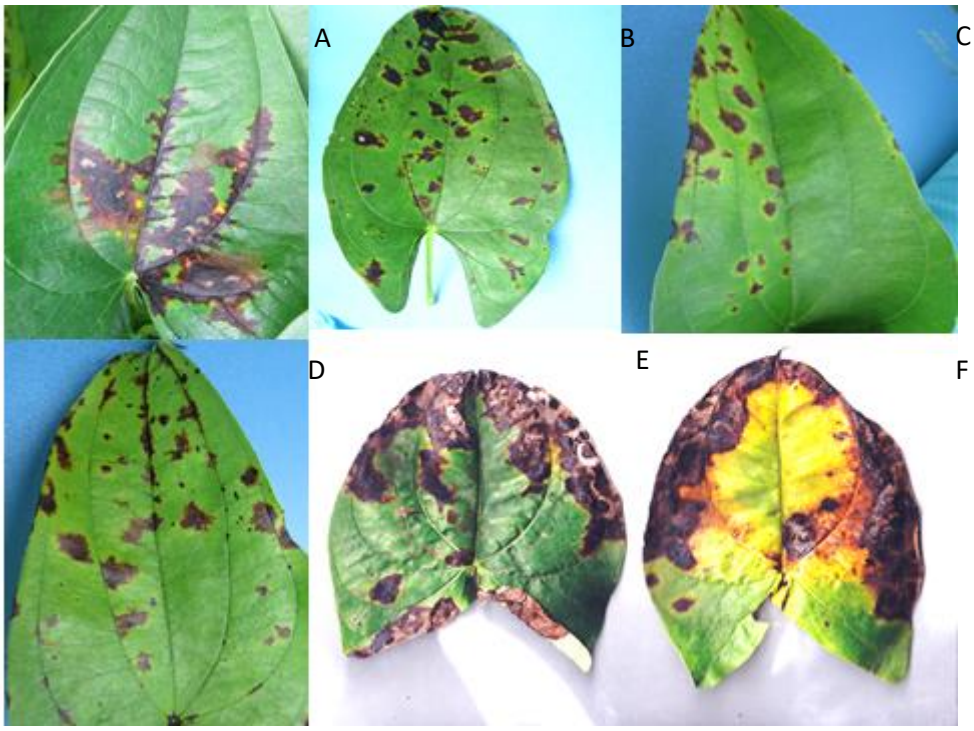

Figure 2. YAD symptoms on *D.alata* leaves (A = blight spreading along the veins, B, C and D= Spots, E= blight and F=blight on an old leaf)

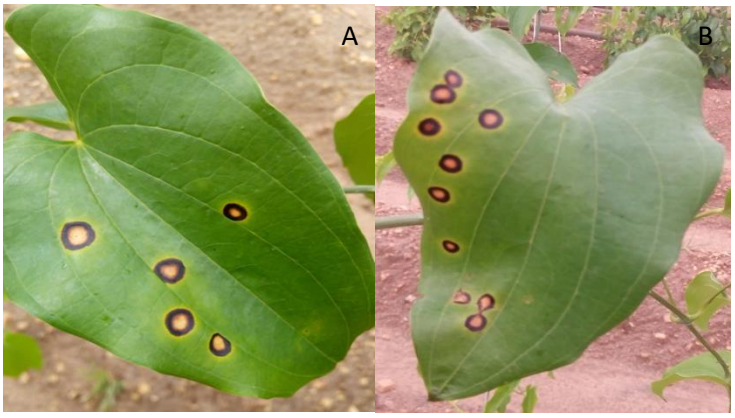

Figure 3. Spot symptoms on *D. rotundata* leaves

Laboratory and field protocols

List of basic laboratory equipment needed for resistance screening

1. Lamina flow hood
2. Petri plates
3. Bunsen flame or ethanol lamp
4. Wash bottle
5. Gloves
6. Incubator
7. Hemocytometer
8. Tele counter
9. Inoculating needle/loop
10. Microscope
11. Surgical blade and holder
12. Hand held/ Spraying can/jar/atomizer
13. Beaker/Conical flask
14 Mira cloth/ strainer / cheese cloth
15 Microscopic slides
16. Cover slip
17. Vials
18 Paper towel
19. Parafilm

List of chemicals needed for resistance screening

1. Microfuge tubes.
2. Glycerol
3. PDA medium
4. Tween 20 or Tween 80
5. Sodium hypochlorite
6. Lactic acid
7. Ethanol
8. Sterile distilled water

Preparation of Potato Dextrose agar (PDA) media

Microorganisms can be grown in vitro for use in genetic and epidemiological studies which entails their isolation, purification and identification. This medium provides common nutrients which are found to be required in all living things such as carbon, nitrogen, sulfur, phosphorus, potassium, magnesium, calcium, oxygen, iron and additional trace elements. These are present as micro and macro elements. Both macro and micro nutrients are critical in proper organismal growth as they play important roles in cellular and metabolic processes. Each nutrient is essential for growth and based on its concentration and presence or absence can control growth. *C. gloeosporioides* grows well on PDA media.

- Weigh 39 g per liter of Potato dextrose agar with the aid of weighing boat and spatula on a weighing balance
- Pour into a clean Duran bottle
- Measure 1000 ml of distilled water
- Add the distilled water to the powder in the Duran bottle
- Allow the mixture to homogenize by heating it slowly at 30^{o}C inside a water bath or hot plate with magnetic stirrer.
- Heat at this temperature until the entire solute dissolves.
- Sterilize it by autoclaving it at 121^{o}C for 15 mins in an autoclaving machine
- Allow it to cool to about 45^{o}C
- Add 1ml per liter of lactic acid or 1g per liter of streptomycin sulphate
- Pour it into 9 cm sterilized petri dishes or glass plate; allow it to solidify or gel.

Sampling techniques

While screening samples on a large field for resistance, our main target is to identify those without so much symptoms and those who are totally symptom free. However, diseased samples must be collected from diverse kinds of symptoms for pathogen characterization. For example, different strains of organism can show symptoms of either spot or blight in which case the two isolates will be obtained, characterized and kept for future use. The samples must be well labelled. Host identity, location, name of collector, type of symptom and date should be written on sample bags or envelopes used for collection.

Isolation and organism identification

Isolation of organisms is key to pathogen identification; it involves the sampling of infected samples such as leaf samples, their surface disinfection and the plating and culturing of organisms in suitable medium. After growing the organism in optimal environmental conditions, the organism is identified under the microscope based on the morphology and growth pattern.

Procedure:

1. Harvest leaves of both types of symptoms of yam anthracnose disease (spot and blight)
2. Sterilize work surface and hands with ethanol. (Caution : Ethanol is highly flammable)
3. Light the lamp and sterilize the scalpel and needle, flame until red hot
4. Check the leaf under the light microscope or stereomicroscope
5. Cut the infected leaf into small pieces and culture the cut leaf itself directly on the PDA_lactic acid medium (up to 5 different surfaces on the plate)
   Or Pick the spores on infected part close to the living portion of plants and place on the PDA medium's surface (Lactic acid acts as a selective antibiotic)
6. Label the plates with the date , the source and the name, seal the plate with parafilm
7. Incubate at 28^{o}C for 4-7days

8. Confirm that it is *Colletotrichum gloeosporioides* (morphological characteristics: form, fluffiness, hyphae structure, type of spores etc.). The organisms vary in color, hyphae type and in their growth rate. They can thereafter be classified based on these criteria.

Preparation of Pure Culture

A pure culture can be obtained from isolating single colony from the starter culture; this enhances homogeneity of the cultures.

The growths observed on the aforementioned PDA media are thereafter transferred to fresh PDA as single cultures as follows:

1. Pick a single colony from the starter culture unto 2 ml of water placed in a sterile petri plate
2. Crush, mix very well, place and spread this mixture on a fresh PDA plate uniformly, seal and label
3. Leave to grow for about 8-14 days at 28^{o}C

Preparation of inoculum

Inoculum is a substance used for inoculation. Usually the causative pathogen is prepared in a solution and applied to an organism in order to infect such organisms. This is usually done in order to screen for resistance to such diseases. The concentration and the mode of application should be optimal for the pathogen in question. The preparation is as follows:

1. Open the 7-14 days old culture and flood with sterile water
2. Scrape mycelia off the medium to release spores
3. Filter the mixture and check for the concentration using the haemocytometer
4. Dilute to the required concentration, usually 10^6 per ml

Storage techniques (Long, medium and short term)

It is difficult to maintain the pathogenicity, virulence and sporulating ability of phytopathogenic fungi if it is not well stored and maintained. Samples of isolates obtained from the field should therefore be well conserved for future use. The cultured isolates can be kept on slants or in glycerol in a -80^{o}C freezer (long term) or -20^{o}C for medium term storage. Short term storage should be done at 4^{o}C.

Procedure for long term low temperature storage

- Prepare 70% glycerol (mix 30ml of water with 70ml of glycerol).
- Homogenize mixture and dispense into 1ml micro tubes.

Note: The tubes must be sterilized in the autoclave at 121^{0}C for 15 mins.

- Purify each isolate to obtain pure culture after 6 days of incubation.
- Reconfirm the identity of each isolate using conventional means of identification considering basic keys (morphological growth, spore formation etc)
- Cut five culture discs with a scalpel blade and transfer into each micro tubes containing 70% glycerol

Note: Each isolate should be replicated for storage at three different temperatures (4^{o}C and -20^{o}C and -80^{o}C)

Rapid screening methods /inoculation techniques

Different methods have been proposed for rapid screening of plants for anthracnose resistance. The most important characteristic of any rapid selection technique is that it should mimic the infection process under natural conditions. The screening method should also be quick, economical and repeatable (Galvan, 2010).

The detached leaf assay is one of such screening methods that have been found to be a good estimate of what happens on the field. The whole plant assay is also used for resistance screening.

Detached leaf method

This involves the cutting of leaf from the growing plant (about 90days old) to see how they react to inoculation with the causative pathogen of anthracnose, *Colletotrichum gloeosporioides.* The reaction has been found to be predictive of what happens in the field and therefore used for rapid screening. The procedure is as follows:

1. Detach leaves from healthy plants
2. Surface sterilize using 1% hypochlorite (30% bleach) for 2 mins
3. Pass through 3-4 rinses of distilled water for 1 min each
4. Wipe off excess water with sterile paper
5. Place on a filter paper moistened with distilled water (with abaxial surface up).
   Caution: Avoid piercing the leaf surfaces with scalpel or forceps
6. Put drops of the inoculum on the abaxial surface (You can also spray using a hand sprayer with or apply with a paint brush). Drops are better for you to ascertain that disease is progressing from point of inoculation.
7. Incubate at 28^{o}C and score at 7 days, 14 days and 21 days. The effect can be measured using digital imaging or visual scoring.

Whole plant assay

This is done by inoculating all the leaves on a growing plant in the screen house or in a sick plot; the seedlings are sprayed using an atomizer with inoculum from the causal pathogen.

1. Put the suspension inside the spraying jar; add 2-3 drops of tween 20 or tween 80 to 500ml of inoculum
2. Spray both surfaces of the leaf especially the abaxial surface of the plant using an atomizer
3. In the screen house, cover with a nylon sheet to create a humid environment or put in humidity chamber of about 80-100% relative humidity.
4. Allow to be there for at least 3 days and then leave in the open (without the nylon) for the symptoms to start being expressed.
5. The symptoms can be scored at 7, 14 and 21days, accessions with very low or no visible symptoms will be selected and subsequently multiplied in a screen house.

Digital imaging techniques (Visible imaging)

This involves the use of optical images for phenotyping. This technique is reliable in estimating disease severity in percentage. Computer software which can recognize and score colour differentiation is used to identify the diseased region relative to the total leaf area. This gives more accurate data. There are free and licensed software that can be used for this type of analysis. Images are scanned into the software and the diseased area is either calculated manually or automatically. Such software includes Image J (free), Leaf doctor (Pethybridge and Nelson, 2015) and Assess 2.0 (Lamari, 2008).

Procedure for the use of Leaf Doctor:

1. Take digital images of leaves to be analyzed from a fixed distance and same exposure and light effect.
2. Analyze the % diseased portion using one of the software.
   (a) Adjust the threshold to separate the infected portion from the diseased free area.
   (b) Measure or analyze In the case of Leaf Doctor, you are able to choose up to 8 points representing the colour of the healthy portion, you will thereafter adjust the threshold and the software will calculate the percentage of the diseased leaf automatically.

Note: Leaf doctor works with a black background while Assess 2.0 ® is best with blue or white background. Pictures can be taken with specified background on the field or adjusted with an image editor such as Adobe Fireworks ® or Magix ®

Alternatively for Assess 2.0

1. Scan the leaves and import pictures into the software for analyses and follow similar steps as described above
   Caution: Use the same specifications on the scanner

   Note: there are other types of digital imaging such as thermal imaging, spectral imaging which are dependent on fluorescence

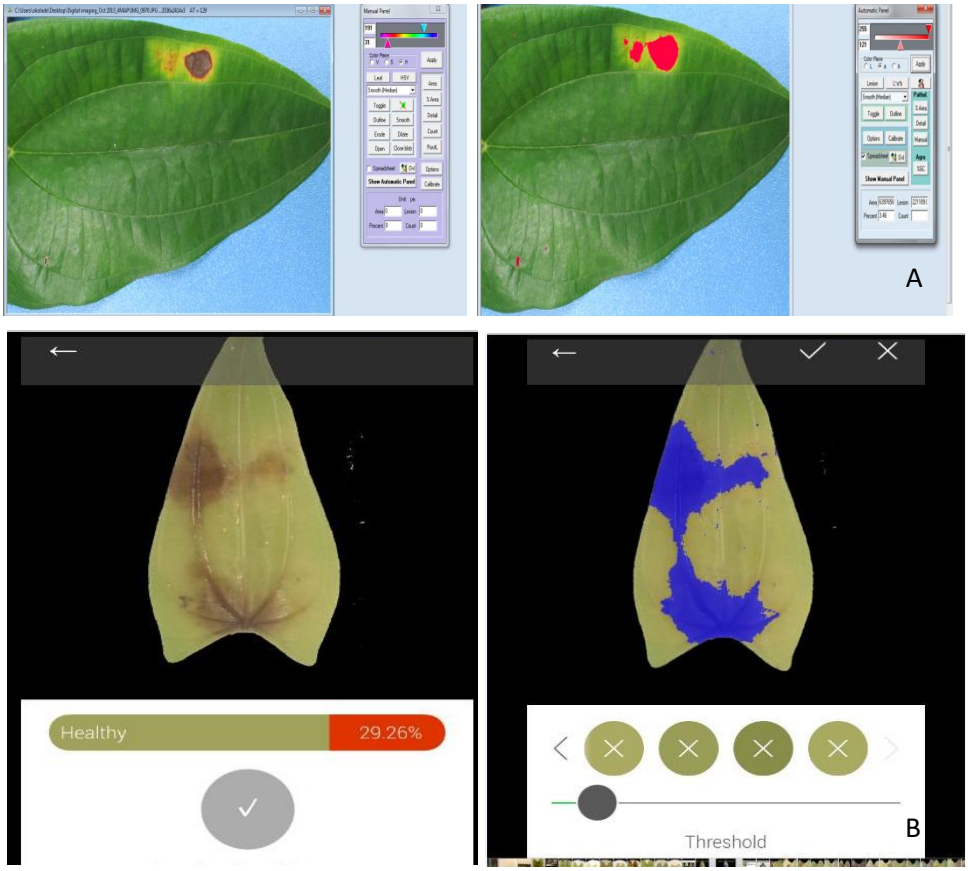

Plate 3: Typical pictures obtained from (A) Assess and (B) Leaf Doctor

Scale of measurement of severity

The use of uniform measuring scale will enhance a relatively fair comparison and harmonization of data collected by different raters

The scale that is being used for the germplasm identification is as follows:

1 = No symptoms and rated as highly resistant

2 = >1-25% area with symptoms and rated as resistant

3 = >25-50% area with symptoms and rated as moderately resistant

4 = > 50%-75% area with leaf symptoms and rated as susceptible

5 = >75% and about dying as a result of attack (Highly susceptible)

Similar or slightly modified version of the scales have been used by Green and Simon (1994), Abang *et al.*, (2002), Onyeka *et al.*, (2006), Aduramigba *et al.*, (2008) and Popoola *et al.*,(2013).

Glossary of terms

Disease scoring

Some of the important measurements which are taken on the field while screening for diseases generally include disease incidence, disease severity, and some factors to consider are resistance and tolerance. The definitions of these terminologies are as follows:

Disease Incidence

This is measure of the proportion of a plant community that is diseased , this works well in cases where the disease is an ‘all or nothing’ type, where any affected plant is inevitably killed, counts of affected plants can allow us to assess the effect of the disease.

Disease severity

This is a measure of the proportion of plant area that is affected and it is mostly useful when the disease is found in varying proportions on the field.

Resistance

The capacity of an organism to defend itself against pathological processes or the agents of those processes

Tolerance

Tolerance is *defined* as the host's ability to offset the negative effects of infection, in this case, the plants recover in terms of symptoms expression

Susceptibility: The state of being easily affected or harmed by a pathogen

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