

Glass house screening for resistance to *Fusarium oxysporum* f. sp. *cubense* - race 1

Introduction

Banana (*Musa* spp.) is an important staple and source of income for many people in developing countries. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) race 1, is a major disease, causing yield losses of up to 100% (Arinaitwe *et al.*, 2019). This is due to narrow or limited genetic base banana cultivars have (Brown *et al.*, 2017), making them highly susceptible to biotic and abiotic stresses (D'Hont *et al.*, 2012). Foc. once introduced into farmer's fields, very little can be done to eradicate the pathogen and there are no effective management strategies. In the past, attempted control measures included injection of chemicals, soil treatments including fumigation and incorporating soil ameliorants/amendments, which were able to reduce the severity of the disease, but none of them was commercially applicable. It is widely reported that the breeding and selection for disease tolerance or resistance is the most effective and sustainable management option available for smallholder farmers (Buddenhagen, 2009). Although several studies confirmed some wild bananas to be important reservoirs of Foc-resistance genes, a more systematic evaluation of wild banana and hybrid species is necessary to generate the information regarding traits and characters on their Foc resistance (Li *et al.*, 2014). Therefore, phenotyping of quantitative disease resistance (QDR) through exposure of plants to pathogens and visual observation of disease symptoms is an important stage in many plant breeding programmes (Skelsey and Newton, 2014).

Bananas is evaluated for Fusarium wilt disease responses in screen house, greenhouse conditions, or in the field. All the practices have advantages and disadvantages, which are largely influenced by the objectives, the number of genotypes to be screened, the time, costs involved and the available facilities. For example, results obtained from pot and field screenings do not always correlate (Ndayihanzamaso *et al.*, 2020). Field evaluation of disease-resistant/tolerant banana plants in soil infested with Foc is highly effective. However, it is slow because disease symptoms usually takes 4-5 months, it is costly and factors affecting disease expression such as inoculum concentration and uniformity, edaphic conditions, temperature and other variables are difficult to control. Greenhouse screen house and glasshouse screening experiments are considered as alternative method for field screening for resistant that will shorten the time, provide uniformity in terms of inoculum concentration, temperature, light/insolation, humidity etc. for example two-month-old plantlets (10-15 cm tall) were reported to be suitable for differential disease symptom expression, and gave similar results to field evaluations at the Fusarium 'hot spot' (<http://www.fao.org/3/ae216e/ae216e0k.htm#fn41>). The bottom line is that

field trials are the final determinants of adaptability and practical functionality of any of these control tactics.

Materials and methods

Plant materials

- 1) TC generated plant material including:
 - a) Test genotypes (newly developed hybrids)
 - b) Resistant checks:
 - i. Calcutta 4
 - ii. KM5
 - iii. CV Rose
 - iv. Kazirakwe
 - c) Susceptible checks:
 - i. Sukali ndiizi - Silk
 - d) Land race controls:
 - i. Matooke
 - ii. Plantain
 - iii. Mchare

Other materials

1. 2Kgs Plastic pot/Polyethylene bags.
2. Autoclave forest soil.
3. Seedling under hardening-off.
4. Millet seeds.
5. Gloves
6. 1L Schott bottle or 250ml Erlenmeyer flasks.
7. Sterile distilled water.
8. Autoclaved paper towel/tissue paper
9. Measuring cylinder (1L)
10. Autoclave.
11. Alcohol (70%)
12. Scalpel/knife.
13. Clean bag/s of 15-20 kgs size

Multiplication, weaning, hardening and transplanting of tissue culture plantlets

Pathogen-free micro-propagated banana plantlets from the TC lab are used for the screening purposes. At 2-3 months post hardened, the plants are transferred to glass house and placed in pots following the adopted experimental design without removing the polythene bags in which they were hardened.

Experimental design

Parental genotypes

Partially replicated experimental design (P-Rep) with three blocks will be adopted with each parental genotype occurring in duplicate and the check

and controls in triplicate for the entire experimental set up. Each plot will constitute two to three plants per genotype. P-rep designs are useful when plant materials and space are limiting factors. Infact, P-rep designs allow for repeated trial evaluations at different locations. The experimental design for the 72 parental genotypes including checks is shown in Table 1 below. The average efficiency 0.99 indicating that the design is optimal.

Table 1. P-rep lay out for banana weevil screening for the available 51 parental lines including checks

Block 1																		
7	2	6	2	9	3	S	1	4	6	4	2	5	5	S	6	4	S	
	8		9		9	6	6	5	0	3		8		1	3	0	4	
6	4	4	5	5	S	5	1	4	2	6	6	3	6	1	2	5	2	
1	8	2	3	6	5	0		7	6	4	6	5	9	4	2	1	5	
2	3	1	6	3	7	5	S	2	S	2	1	3	4	4	1	4	1	
0	6	5	2		1	2	2	4	3	7	8	8	9	1	9	4	1	
Block 2																		
4	4	1	2	2	3	S	5	3	8	2	S	2	1	4	5	S	1	
1	2	0	0		7	6	4	3		4	3	1	5		0	1	6	
5	S	6	S	5	2	5	3	4	1	3	7	6	1	3	4	5	7	
7	2	8	5	6	3	5	9	5	8	0	2	5	2	1	8	1	0	
1	4	2	5	3	6	6	2	2	3	4	4	3	1	S	6	1	3	
7	6	5	9	2	7	1	8	9		3	9	4	1	4	3	3	5	
Block 3																		
1	3	4	7	6	6	5	1	3	4	5	6	1	2	3	2	6	6	
7	4	7	1	7	8		3	8	0	9	0	4	3	1	7	2		
2	3	6	2	8	1	5	1	2	S	1	S	4	5	3	4	7	7	
1	3	6	6		0	4	2	2	2		1	4	8	0	6	2	0	
4	6	5	3	5	3	5	S	S	3	S	1	5	6	S	9	6	7	
	4	7	7	2	6	5	5	3	2	4	9	3	5	6		9		

Hybrids

The augmented design resulting into an incomplete block design where standard checks are replicated in each block and test genotypes will not be adopted for the hybrids. Such augmented designs are very useful where very large numbers of hybrids are produced with limited planting materials and reduced space.

Media preparation and incubation of Foc-race 1

1. Add 39 g of PDA powder (full strength) to 1L Schott bottle and fill up

with deionized water to the 1L mark.

2. Autoclave at 121°C for 20 min; and leave to cool down to 50°C.
3. Dispense the cooled media into 90-mm/60mm Petri dishes and store at 4°C until use.
4. Transfer the Foc isolate onto dextrose agar (PDA) and leave it to germinate on agar for 7 days at 25°C on a bench or in the incubator.

Inoculum preparation

Millet seeds preparation and inoculation of millet seeds

1. Soak millet seeds in a 15-20 kg bag for 6h. Allow the fully soaked millet seeds to dry.
2. Autoclave the millet at 121°C for 2hrs. Allow to cool under the lamina flow hood.
3. Cut mycelia from the margins of a fully grown Foc culture and inoculate the sterilized millet kernels.
4. Thoroughly mix the Foc with the millet and incubate at 25°C in a dark room/incubator.
5. Shake the container to evenly mix the Foc and millet every two days; and to also prevent clumping of the kernels together.
6. After 14 days, individual millet kernels appear discolored with a reddish taint.
7. Plate a small sample from each container onto PDA to confirm that colonization is entirely by Foc.

Inoculation and incubation

1. Thoroughly mix the inoculum and apply 25 gms of Foc-infested millet/1 kg sterilized soil, in a pot pre-filled with 1/3 sterilized soil.
2. Then banana plantlets are planted in the pots while ensuring that roots are in contact with Foc-infested millet-soil mixture. Pots are further filled with the sterilized soil up to 2/3, making a total of 2kgs per pot.
3. Leave the banana plantlets to grow (incubate) in glasshouse on iron bared benches at a temperature range of 20-25°C. Regularly water the plants with tap water.

Assessment

Assessment of Fusarium wilt severity is based on observation of yellowing and wilting of leaves as well as internal corm discolorations. Usually, the degree of leaf symptom expression and rhizome discoloration intensifies with increasing spore concentrations.

External symptom data collection

a) Leaves

During evaluation among important symptom used includes wilting and yellowing of the older leaves around the margins. The yellow leaves may remain erect or collapse at the petiole. Sometimes, the leaves remain green, except for spots on the petiole, but still snap. The collapsed leaves hang

down the pseudo stem like a skirt. Eventually, all the leaves fall and dry up. Other symptoms include irregular, pale margins on new leaves and the wrinkling and distortion of the leaf blade (Fig. 1). The evaluation uses a scale of 1 to 5 as described by (Viljoen et al., 2017). Evaluation is done every week starting the second week post inoculation up to 10-12 weeks.

	<p>1 - No visual leaf symptoms</p>
	<p>2 - 0-33% of older banana leaves turning yellow</p>
	<p>3 - 34-66% of older leaves turning yellow, with some hanging down the pseudo stem</p>
	<p>4 - 76-100% of leaves turning yellow and necrotic, with leaves hanging down the pseudo stem</p>
	<p>5 - Plant dead, with brown leaves hanging down pseudo stem</p>

Figure 1. External banana fusarium wilt disease symptoms and the rating scale of 1 to 5 by Viljoen *et al.*, 2017

b) Pseudo stem

Splitting of the base of the pseudo stem is another common symptom.

2.13. Internal symptom data collection

The characteristic internal symptom of Fusarium wilt is vascular discoloration, which varies from pale yellow in the early stages to dark red or almost black in later stages. Internal symptoms first develop in the feeder roots, which are the initial infection sites. The fungus spreads from the edge toward the inner part of rhizome and then to the pseudo stem. The evaluation of corm is done at the end of the experiment, which is 10 to 12 weeks.

- Cut the corm/rhizome longitudinally into two halves starting from the collar region.
- A scale of 1 - 6 is used to evaluate discoloration of the corm (Fig. 2).

Internal Corm Scores	Description
 Scale 1	Corm completely clean, no vascular discoloration
 Scale 2	Isolated points of discoloration in vascular tissue
 Scale 3	Discoloration of up to one-third (1/3) of vascular tissue
	Discoloration between one-third and two thirds (1/3 - 2/3) of vascular tissue

	
Image 1: Scale 4	Discoloration of greater than two-thirds (2/3) of vascular tissue
	
Image 2: Scale 5	Total discoloration of vascular tissue
	
Scale 6	

Figure 2. Scale used for internal discoloration of the corm for Foc-R1 evaluations

Data analysis

1. To determine the variation among genotypes, analysis of variance is carried out using the following linear model: Genotype response = μ + genotype effect + block effect + block/rep effect + error
2. Both Dunnett's test and fishers protected least significant different test using GenStat are used to separate the resistant genotypes from susceptible genotypes with comparison to the positive and negative checks.

Calculations of Disease Severity Index (DSI)

Disease severity index is a special type of ordinal scale comprising several intervals of known numeric ranges that can be used when estimating severity of a plant disease. Disease severity indices are used to indicate the performance of a cultivar regarding disease resistance at one location under a given set of conditions

$$\text{DSI (\%)} = \frac{[\text{sum (class frequency} \times \text{score of rating class)}]}{[(\text{total number of plants}) \times (\text{maximal disease index})]} \times 100.$$

According to FAO, the DSI consists of four designations, namely resistant, tolerant, susceptible, and highly susceptible (Table 2). If the cultivar is resistant in LSI and tolerant in RDI, the cultivar is considered as tolerant. If RDI is tolerant and LSI is susceptible, the cultivar is susceptible. The final status of the cultivar is considered as resistant when both LSI and RDI for each treatment show resistance. If one of the responses is tolerant, the cultivar is then considered as tolerant.

Table 2. Translation of DSI scales

DSI Scales for LSI	DSI Scales for RDI	Translation
1	1	Resistant
Between 1.1 and 2	Between 1.1 and 3	Tolerant
Between 2.1 and 3	Between 3.1 and 5	Susceptible
Between 3.1 and 4	Between 5.1 and 8	Highly susceptible

Appendix

1. Culture medium preparation materials

- I. PDA half strength/ sabourand Dextrose Agar (SDA).
- II. Deionised water/distilled water.
- III. Petri dishes (90-mm-diameter).
- IV. Analytical balance.
- V. Weighing boats/trays.
- VI. Spatula.
- VII. 1-L Schott bottles.
- VIII. Autoclave.
- IX. Petri-dishes (90mm, 60mm)
- X. Anti-biotics

2. Fusarium diseased sample collection

- The sample is taken from symptomatic plant pseudo stem. Avoid areas where decay is advanced. The chance of recovering healthy cultures of Foc decreases as the sample deteriorates, so avoid sampling too wet samples which attract bacteria.
- Vascular bundles/Xylem strands are keenly cut from the leaf sheath and put in between sterile paper towel and squeezed to remove excess water. Samples should be kept in sterile paper towel and paper bags or wrapped in paper until the strands dry.
- Avoid plastic bags as this causes the samples to sweat and promotes growth of bacteria.
- Label the sample properly and store in a dry shelf.

3. Fungal isolation from affected colored strands.

- Isolation can be attempted as soon as the strands with vessels are dry.

- Plate small sections (3-6 mm long) of the tissues with vascular vessels in Petri plates with antibacterial agent (i.e. streptomycin sulfate 1.2 mL / 240 mL of full strength PDA).
- Check if there is growth of fusarium from 2 to 4 days.
- Fungal growth can easily be masked if there is bacterial contamination. Allow the sample to dry further and increase the streptomycin sulfate in the media.
- From samples that have been prepared correctly, a high rate of Fusarium recovery is possible.
- Then you can do single spore by picking a single conidia/spore from each specimen.

4. Isolation from soil

- Collect a soil sample from the first 25 cm depth and store in a paper bag.
- Let samples air dry in the more aseptic conditions for 24-48 hours
- Grind the larger particles in a mortar
- Prepare a soil suspension in sterile water in a proportion of 1:50 soil weight / water volume (If the suspension is too concentrated because of high Fusarium population in the sample, a 1:100 proportion can be prepared).
- Shake the suspension for better release and distribution of soil particles and fungal structures.
- Dilute 1 mL suspension in 10 cm Petri plates with modified K2 media at close to melting temperature to achieve a good dispersion of the soil in the culture media.
- 1 mL of suspension can also be distributed on the surface of the plate with solidified K2 media.
- Agar should be allowed to dry for 3-4 days in the plates before plating the spore suspension so that it can absorb a higher amount of the spore suspension.
- Distribute the suspension as uniformly possible and allow it to stand for two minutes. Remove excess soil suspension from the plate and incubate it at 27°C upside down.
- Recovered colonies are transferred to other appropriate media to obtain single conidial isolates.

5. Single spore isolations (single conidia)

- Foc single spore isolations are obtained by the plate dilution method and streaking plates (showed ahead).
- For both methods: Collect a scrape of sporulating hyphae from cultures growing on PDA (¼ strength) and dissolve in 10 mL sterile distilled water in test tubes.
- From an initial suspension, a dilution serial can be prepared.
- Pipette or streak 1 mL of each of the dilutions on water agar.
- Using a stereo microscope pick and incubate a single spore on PDA.

6. Short term storage of Foc cultures

- Store cultures in a 4°C refrigerator and regularly subcultured.

7. Long term storage of Foc cultures

- 10 mL of 15% glycerol is pipetted over the fungal growth in a Petri plate in a sterile air flow bench cabin. The spores and some hyphae are released gently with a sterile and cool scalpel
- Aliquots of 1mL are pipette into 2 mL cryovials tubes.
- Each one of the cryovials is carefully marked and stored in cryo-boxes at -80°C.

8. Reviving Foc culture

- Carnation leaves are used for revitalizing the isolate by adding few pieces of leaves into the media during the cooling time after sterilizing the media.
- Four to ten sterilized pieces of carnation leaves are placed on water agar/PDA surface before media hardens (solidifies).
- After the media has solidified, the plates with CLA are stored in a refrigerator at 4°C.

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