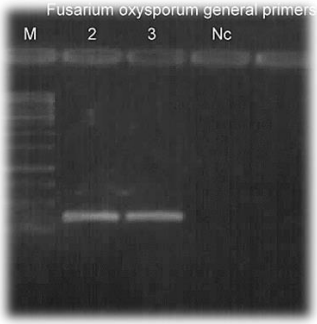
Novel Approaches to the Improvement of Banana Production in Eastern Africa - the application of biotechnological methodologies



NARO-Bioversity Banana Improvement Project

Technical Report , 1 July 2010 to 30 June 2021

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1 Executive Summary

The project's overall objective is to to build capacity for the development and deployment of biotechnological tools and products critical for the transformation of Uganda's agricultural economy. The focus is to improve resistance to pests (weevils, nematodes) and diseases (Fusarium wilt) in the East African highland bananas through the use of novel biotechnological approaches. The project is in Phase IV whose activities and objectives build on the achievements of previous phases. For example, Phase III objectives included; (i) Capacity building – human and infrastructure with emphasis on building a cadre of experts and critical infrastructure to support research and development innovations, (ii) Technology transfer - Access and apply technologies and (iii) Build partnerships and networks.

In addition to the outputs and successes of Phase III, the end of Phase III Project reported identified gaps such as; (a) Products developed need to be advanced for de-regulation and release. The transgenic banana lines were screened under screen house conditions resistance to weevils and nematodes and were selected for testing under confined field trial (CFT) conditions, (b) Gene mining for useful traits for now and the future and (c) Building capacity for gene editing. Therefore, these gaps presented opportunities that shaped the objectives of Phase IV. Therefore the objectives of Phase IV are; (i) To evaluate and release nematode and weevil resistant banana products and a tracking system and (ii) To generate new technologies (genes) for Foc resistance and develop resistant dessert bananas.

Notably, Phase IV commenced in January 2020 and despite a delay in release of funding and commencement of project activities, this report highlights and emphasises research outputs and progress achieved in Year one period (January to December, 2020) of Phase IV of the Biotech project.

2 Progress of Year 1

2.1 Objective 1: Evaluate and Release nematode and weevil resistant banana products and a tracking system.

2.1.1 Activity 1.1 Select elite lines from CFT at Kawanda

To achieve this, the following sub-activities were proposed (i) Establish and maintain plants in glass and screen house facilities, (ii) Propose CFT site for NBC approval, (iii) CFT Application submitted and NBC approved, (iv) Establishment of CFT facilities at NARL, Kawanda, (v) Inspection and approval of CFT facilities by NBC, (vi) Collect, establish and maintain nematode inoculum in potted plants, (vii) Collect, establish and maintain weevil inoculum in potted plants, (viii) Nematode inoculation (Pre-planting), (ix) Planting of CFT at NARL, Kawanda and (x) Weevil inoculation (Post-planting).

2.1.1.1 Activity 1.1.1: Establish and maintain plants in glass and screen house facilities

Prior to Confined Field trial (CFT) transfer of transgenic bananas developed in Phase III, multi-copies of selected lines of 'Nakitembe' (NKT) and 'Gonja Nakatansense' for weevil and nematode resistance were established and maintained under screen house conditions. This work explores the application of insecticidal and nematidical properties of *Bacillus thuringiensis* (Bt) crystalline (cry) proteins and plant proteinase inhibitors (cystatins) in the control of weevils and nematodes which are major pests of bananas in Uganda. As such, transgenic banana lines with single and stacked gene constructs of Bt *Cry6A*, *Carica papaya* cystatin (*CpCYSΔ89*) and their respective transgenic and non-transgenic control lines (Fig. 2.1; Table 2.1) were maintained in pots under screen and glasshouse conditions and later planted in the approved CFT site for further evaluation under field conditions.

Figure 2:1: Ready to plant transgenic lines (A; cut back, B; re-established) in pots being maintained under screenhouse and glasshouse conditions.

2.1.1.2 Activity 1.1.2: Establishment of confined field trial facilities at NARL, Kawanda

On June 16th, 2018, the Project was granted permission to plant the selected transgenic lines under field conditions in a confined field trial (CFT) under Application Reference Number: NBC/01/2018 with a UNCST Decision Number 1/2018. To facility the establishment of the CFT the Project's proposed site was approved to by the NBC to host the CFT in accordance with the Standard Operating Procedures (SOPs) and National Guidelines for Field Trials of Genetically Engineered Crops by Uganda National Council for Science and Technology (UNCST). Prior to construction of regulatory facilities, the site was fenced off with chain-link and later divided into two experimental fields to facilitate testing bananas for resistance to nematodes and weevils respectively (Fig. 2.2).

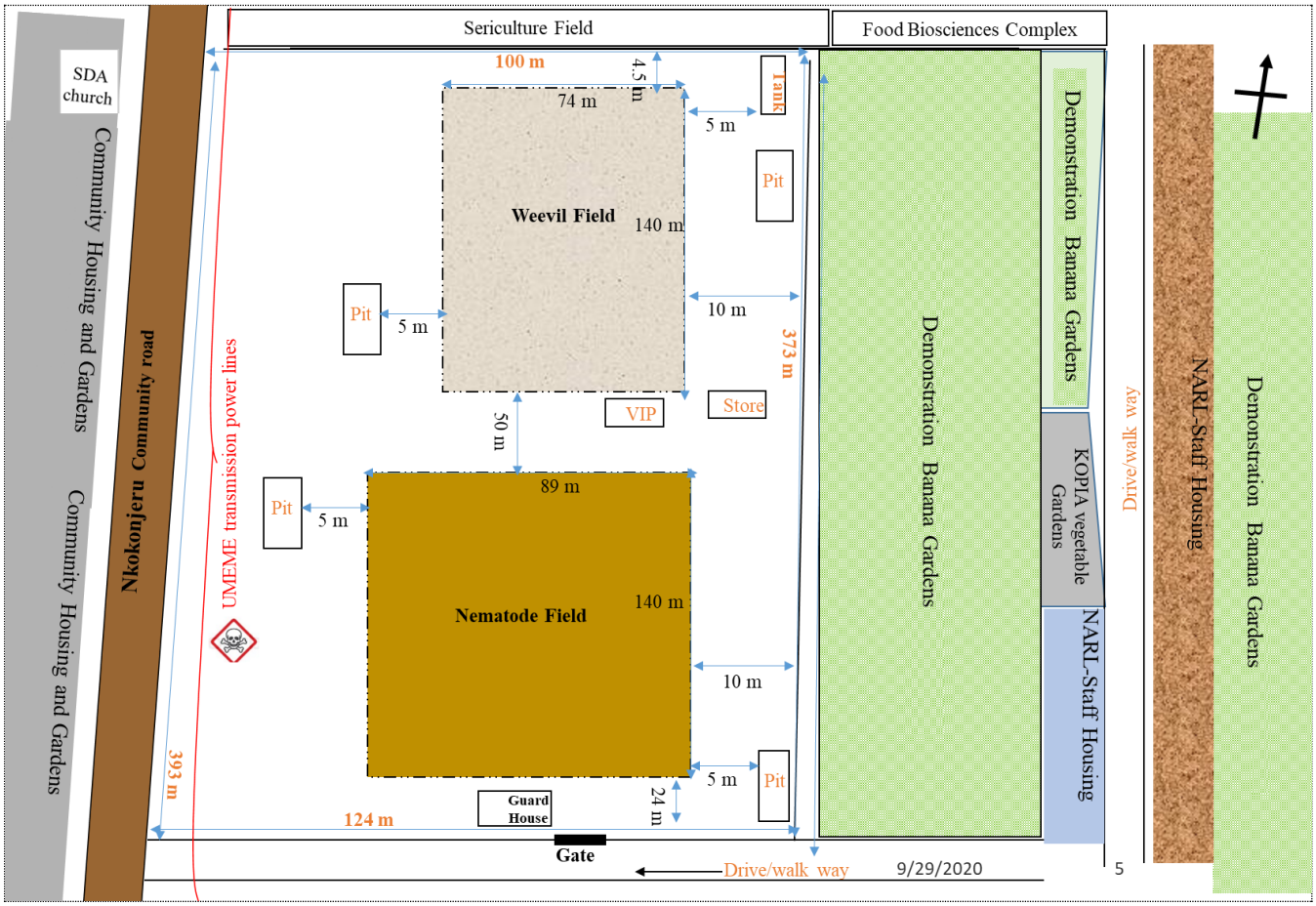


Figure 2:2: The CFT site with the layout of regulatory facilities, water tank and the two fields.

To meet regulatory compliance, the Project constructed in-site facilities such as (i) 24/7 security surveillance with 4 CFT security personnel and guard house with equipment storage partition, (ii) cleared overgrown bush, dug and marked planting holes in the CFT site, (iii) procured garden tools and chemicals

to be used in the site, (iv) constructed four incineration pits for disposal of in-field transgenic waste, (v) constructed sanitation facility and (vi) printed a regulatory sign post to CFT site and in-field plant labels. Additionally, the Project (vii) procured manure to improve soil fertility and (viii) installed a water supply system including a 10,000 litre reservoir raised on metallic stands connected to a mains 800 metres away to supply water to the in - CFT site plants via a network of 6 water taps upstream of the tank was established (Fig. 2.3). Upon completion of the regulatory facilities, a pre-planting inspection of the site was conducted on October 14th, 2020 and with no objection was subsequently approved as a CFT by the NBC to host transgenic plants.

Figure 2:3: Location of the CFT site and its regulatory facilities at NARL, Kawanda.

2.1.1.3 Activity 1.1.3: Maintenance of inoculum for banana nematode populations

Potted plants selected for resistance to nematodes were pre-inoculated with pure nematode cultures of *Radopholus similis* (918 nematodes/plant) into their root system one month to planting to allow proper establishment of inoculum. Since over 2,000,000 nematodes were needed, the Project initiated and maintained pure *R. similis* cultures *in vitro* (on carrot discs) and *in vivo* (in roots of potted selected East African highland banana cultivars in the screen house) (Fig. 2.4).

Figure 2:4: Maintenance of nematode inoculum *in vitro* on carrot discs (A) and *in vivo* in banana roots (B) and pre-planting inoculation of selected transgenic lines (C, D).

2.1.1.4 Activity 1.1.4: Environmental Impact Assessment

Biotic indicators (fungi, bacteria, algae, protozoa and nematodes) of soil can be used to assess changes in soil condition due to land use and management practices. Nematodes can act as bio-indicators of environmental safety. This is attributed to nematodes having several biological features that reinforce their use as bio-indicators and therefore can be used to determine environmental health. Nematodes have; (i) permeable cuticle which allows them respond to range of reactions to pollutants, (ii) resistant stages - cryptobiosis / cysts to survive inactively adverse environments, (iii) heat shock proteins whose expression is enhanced when exposed to heat, metal ions, or organic toxins.

Therefore, prior to planting of the transgenic banana lines, the Project established the baseline status of nematode populations based on morphological properties. Soil samples from the CFT site were analysed to identify and quantify parasitic and non-parasitic nematode species. This baseline data will also provide an indicator on the effect of introduced transgenes to non-target organisms. Based on feeding groups, the pre-planting ecological status of the soil environment in the CFT was characterised by majority of

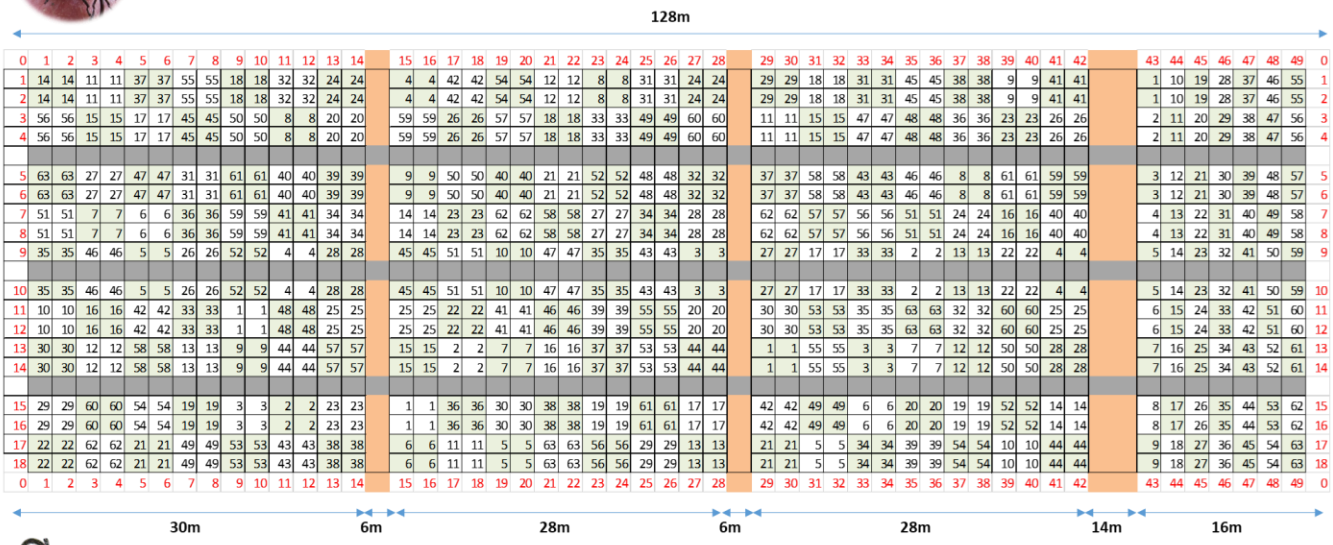
non-parasitic nematode genus as bacterivores (*Acrobeloides*, *Heterocephalobus*, *Cephalobus*), followed by fungivores (*Aphelenchus*) and omnivores (*Mesodorylaimus*, *Dorylaimoides*). Additionally, parasitic nematodes included *Rotylenchulus reniformis*, *Helicotylenchus dihystera*, *Scutellonema sp.* and *Tylenchulus semipenetrans*. Notably, the field did not show presence of *R. similis*, plant parasitic nematodes for bananas which the transgenic technology is targeting.

This baseline will be repeated at the end of the CFT to verify any changes in nematode groups, species and abundance as a measure of ecological status of soil being an indicator of environment stability. Since the method of assessment was based on morphological features of nematodes, the Project proposes to utilise molecular tools especially, using heat shock proteins as biomarkers to study nematodes as bio-indicators for assessment of eco-toxicology of soils (Kammenga *et al*., 2000) and therefore environmental safety.

2.1.1.5 Activity 1.1.5: Planting of banana transgenic lines in the confined field trial at NARL, Kawanda

The CFT site was divided into two fields, one for evaluating plants for resistance to nematodes (140 metres by 89 metres; 1,286 holes) and another for weevils (140 metres by 74 metres; 1,008 holes) whose respective layouts and planting field maps were developed and holes marked with respective CFT plant number (Fig. 2.5; Tables 2.1, 2.2). The field was planted in RCBD with each line represented by 12 clones, planted in square plots of 4 clones per line, and each plot replicated in 3 blocks. Each field had a fourth block with plots of two plants per line representing the non-inoculated plants. Plant spacing in both fields is 2 X 3 metres between rows and columns respectively. Nematode resistance lines of NKT were 24 *Cyst*, 19 *Cry6A,* 16 *Cyst*+*Cry6A* and 1 *GUS* transgenic lines plus 1 non-transgenic while Gonja had 6 *Cyst*, 3 *Cry6A* and 12 *Cyst*+*Cry6A* with 1 KM5 as resistant wild-type control. Additionally, weevil resistance lines included transgenic NKT with 5 *Cyst*, 25 *Cry6A,* 10 *Cyst*+*Cry6A*, 1 *GUS* and 1 non-transgenic NKT while Gonja had 13 *Cyst*, 2 *Cry6A*, 5 *Cyst*+*Cry6A* and 1 resistant wild-type control KM5. Planting of the 83 nematode resistant lines concluded on December 1st, 2020 while December 4th, 2020 for the 63 weevil resistant lines (Fig. 2.6).

Weevil Field



Nematode Field

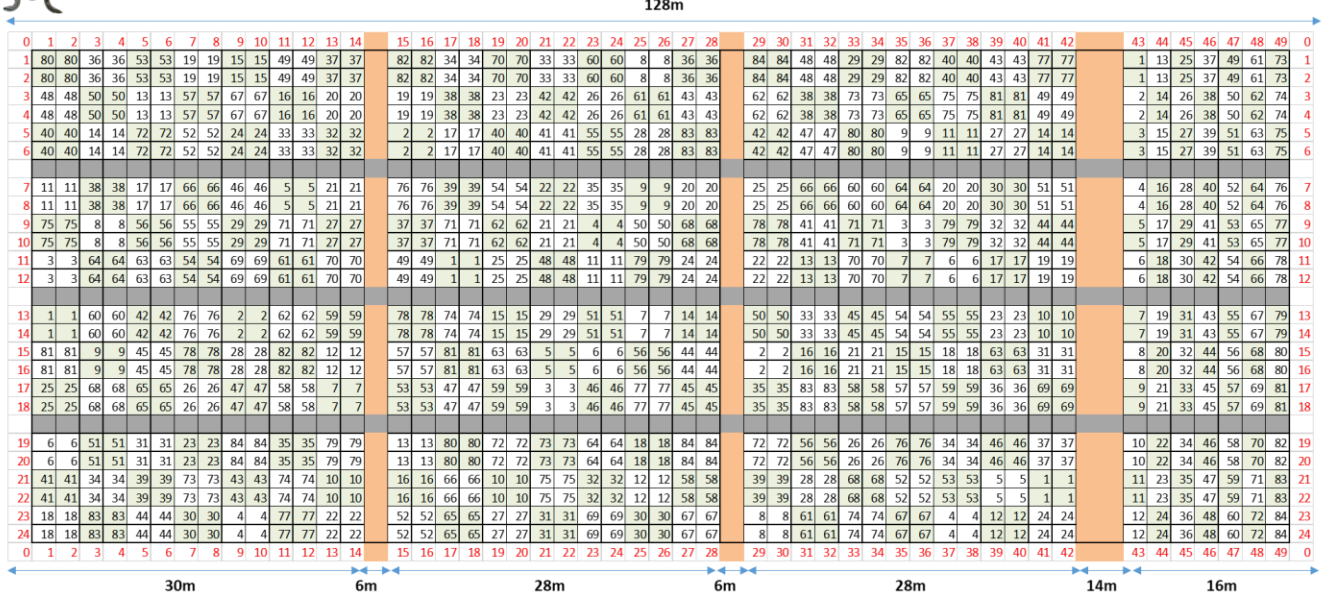


Figure 2:5: The layout of the planting map of the two respective fields in the CFT site at NARL, Kawanda.

Table 2:1: Selected transgenic banana lines (for nematode field) planted in the CFT

Serial No.	CFT Number	Cultivar	Gene	Pest	Copies plante in CFT	Serial No.	CFT Number	Cultivar	Gene	Pest	Copies plante in CFT
1	237039	NKT	CRY6A	Nematode	14	41	247003	NKT	CYST	Nematode	14
2	137045	Gonja	CRY6A	Nematode	14	42	247005	NKT	CYST	Nematode	14
3	137061	Gonja	CRY6A	Nematode	14	43	247008	NKT	CYST	Nematode	14
4	137096	Gonja	CRY6A	Nematode	14	44	247027	NKT	CYST	Nematode	14
5	237097	NKT	CRY6A	Nematode	14	45	247028	NKT	CYST	Nematode	14
6	147016	Gonja	CYST	Nematode	14	46	247034	NKT	CYST	Nematode	14
7	147033	Gonja	CYST	Nematode	14	47	247035	NKT	CYST	Nematode	11
8	147035	Gonja	CYST	Nematode	14	48	247052	NKT	CYST	Nematode	14
9	147037	Gonja	CYST	Nematode	14	49	247055	NKT	CYST	Nematode	14
10	147050	Gonja	CYST	Nematode	14	50	247060	NKT	CYST	Nematode	6
11	147139	Gonja	CYST	Nematode	14	51	247086	NKT	CYST	Nematode	14
12	157005	Gonja	CRY6A/CYST	Nematode	14	52	247091	NKT	CYST	Nematode	14
13	157017	Gonja	CRY6A/CYST	Nematode	12	53	247097	NKT	CYST	Nematode	14
14	157020	Gonja	CRY6A/CYST	Nematode	14	54	247109	NKT	CYST	Nematode	14
15	157023	Gonja	CRY6A/CYST	Nematode	14	55	247115	NKT	CYST	Nematode	14
16	157026	Gonja	CRY6A/CYST	Nematode	14	56	247120	NKT	CYST	Nematode	14
17	157027	Gonja	CRY6A/CYST	Nematode	8	57	247123	NKT	CYST	Nematode	14
18	157048	Gonja	CRY6A/CYST	Nematode	14	58	247144	NKT	CYST	Nematode	10
19	157057	Gonja	CRY6A/CYST	Nematode	14	59	247154	NKT	CYST	Nematode	14
20	157096	Gonja	CRY6A/CYST	Nematode	14	60	247164	NKT	CYST	Nematode	14
21	157115	Gonja	CRY6A/CYST	Nematode	14	61	247171	NKT	CYST	Nematode	14
22	157116	Gonja	CRY6A/CYST	Nematode	14	62	247177	NKT	CYST	Nematode	14
23	157134	Gonja	CRY6A/CYST	Nematode	14	63	247178	NKT	CYST	Nematode	14
24	237010	NKT	CRY6A	Nematode	14	64	247179	NKT	CYST	Nematode	14
25	237015	NKT	CRY6A	Nematode	14	65	257018	NKT	CRY6A/CYST	Nematode	14
26	237018	NKT	CRY6A	Nematode	12	66	257031	NKT	CRY6A/CYST	Nematode	11
27	237038	NKT	CRY6A	Nematode	14	67	257035	NKT	CRY6A/CYST	Nematode	14
28	237039	NKT	CRY6A	Nematode	14	68	257036	NKT	CRY6A/CYST	Nematode	14
29	237049	NKT	CRY6A	Nematode	14	69	257038	NKT	CRY6A/CYST	Nematode	14
30	237090	NKT	CRY6A	Nematode	12	70	257044	NKT	CRY6A/CYST	Nematode	14
31	237097	NKT	CRY6A	Nematode	14	71	257050	NKT	CRY6A/CYST	Nematode	14
32	237116	NKT	CRY6A	Nematode	14	72	257051	NKT	CRY6A/CYST	Nematode	14
33	237140	NKT	CRY6A	Nematode	14	73	257071	NKT	CRY6A/CYST	Nematode	14
34	237161	NKT	CRY6A	Nematode	12	74	257105	NKT	CRY6A/CYST	Nematode	14
35	237176	NKT	CRY6A	Nematode	14	75	257120	NKT	CRY6A/CYST	Nematode	14
36	237189	NKT	CRY6A	Nematode	14	76	257127	NKT	CRY6A/CYST	Nematode	14
37	237191	NKT	CRY6A	Nematode	14	77	257131	NKT	CRY6A/CYST	Nematode	14
38	237202	NKT	CRY6A	Nematode	14	78	257142	NKT	CRY6A/CYST	Nematode	14
39	237242	NKT	CRY6A	Nematode	14	79	257152	NKT	CRY6A/CYST	Nematode	14
40	237248	NKT	CRY6A	Nematode	14	80	257153	NKT	CRY6A/CYST	Nematode	14
						81	KM5	KM5	NTC		14
						82	NKT-GUS	NKT	GUS		14
						83	NKT-NTC	NKT	NTC		14

Table 2:2: Selected transgenic banana lines (for weevil field) planted in the CFT

Serial No.	CFT No.	Cultivar	Gene	Pest	Copies planted in CFT	Serial No.	CFT No.	Cultivar	Gene	Pest	Copies planted in CFT
1	138074	Gonja	CRY6A	weevil	14	33	238149	NKT	CRY6A	Weevil	14
2	138090	Gonja	CRY6A	weevil	14	34	238151	NKT	CRY6A	Weevil	11
3	148012	Gonja	CYST	weevil	14	35	238158	NKT	CRY6A	Weevil	14
4	148039	Gonja	CYST	weevil	14	36	238182	NKT	CRY6A	Weevil	14
5	148044	Gonja	CYST	weevil	14	37	238187	NKT	CRY6A	Weevil	14
6	148048	Gonja	CYST	weevil	14	38	238189	NKT	CRY6A	Weevil	14
7	148050	Gonja	CYST	weevil	14	39	238194	NKT	CRY6A	Weevil	14
8	148056	Gonja	CYST	weevil	14	40	238197	NKT	CRY6A	Weevil	14
9	148072	Gonja	CYST	weevil	14	41	238212	NKT	CRY6A	Weevil	13
10	148118	Gonja	CYST	weevil	14	42	238231	NKT	CRY6A	Weevil	14
11	148137	Gonja	CYST	weevil	14	43	238242	NKT	CRY6A	Weevil	14
12	148138	Gonja	CYST	weevil	8	44	238256	NKT	CRY6A	Weevil	14
13	148144	Gonja	CYST	weevil	11	45	238270	NKT	CRY6A	Weevil	11
14	148179	Gonja	CYST	weevil	14	46	248050	NKT	CYST	Weevil	14
15	148189	Gonja	CYST	weevil	14	47	248058	NKT	CYST	Weevil	14
16	158014	Gonja	CRY6A/CYST	weevil	14	48	248090	NKT	CYST	Weevil	14
17	158024	Gonja	CRY6A/CYST	weevil	14	49	248168	NKT	CYST	Weevil	14
18	158101	Gonja	CRY6A/CYST	weevil	14	50	248288	NKT	CYST	Weevil	14
19	158118	Gonja	CRY6A/CYST	weevil	12	51	258028	NKT	CRY6A/CYST	Weevil	14
20	158264	Gonja	CRY6A/CYST	weevil	9	52	258052	NKT	CRY6A/CYST	Weevil	14
21	238043	NKT	CRY6A	Weevil	14	53	258107	NKT	CRY6A/CYST	Weevil	14
22	238046	NKT	CRY6A	Weevil	7	54	258152	NKT	CRY6A/CYST	Weevil	14
23	238051	NKT	CRY6A	Weevil	14	55	258157	NKT	CRY6A/CYST	Weevil	14
24	238053	NKT	CRY6A	Weevil	14	56	258159	NKT	CRY6A/CYST	Weevil	13
25	238063	NKT	CRY6A	Weevil	14	57	258181	NKT	CRY6A/CYST	Weevil	14
26	238070	NKT	CRY6A	Weevil	14	58	258212	NKT	CRY6A/CYST	Weevil	14
27	238090	NKT	CRY6A	Weevil	14	59	258216	NKT	CRY6A/CYST	Weevil	14
28	238096	NKT	CRY6A	Weevil	14	60	258220	NKT	CRY6A/CYST	Weevil	14
29	238112	NKT	CRY6A	Weevil	14	61	KM5	KM5	NTC		14
30	238117	NKT	CRY6A	Weevil	11	62	NKT-GUS	NKT	GUS		14
31	238143	NKT	CRY6A	Weevil	14	63	NKT-NTC	NKT	NTC		14
32	238145	NKT	CRY6A	Weevil	14						

Figure 2:6: Potted plants prior to transportation to CFT (A) and planting of transgenic plants in CFT (B).

2.1.1.6 Activity 1.1.6: Collect, establish and maintain weevil inoculum in potted plants, and Weevil inoculation (Post-planting).

At 6.5 months after establishment of the plants in the CFT, the Project team inoculated each test plant with 10 weevils. This necessitated the Project to rear and maintain 20,000 weevils (*Cosmopolites sordidus*) on-station in the Entomology Lab. Weevils were trapped, sexed, reared on banana corms, cleaned and induced to hatch to new weevils (Fig. 2.7).

Figure 2:7: In-field trapping, sexing and maintenance of banana weevils (A-C) and post-planting inoculation (D-F).

The plants in the CFT have been well maintained through manuring to improve soil nutrition, weeding and mulching for weed control and trenching for soil and water management (Fig. 2.8).

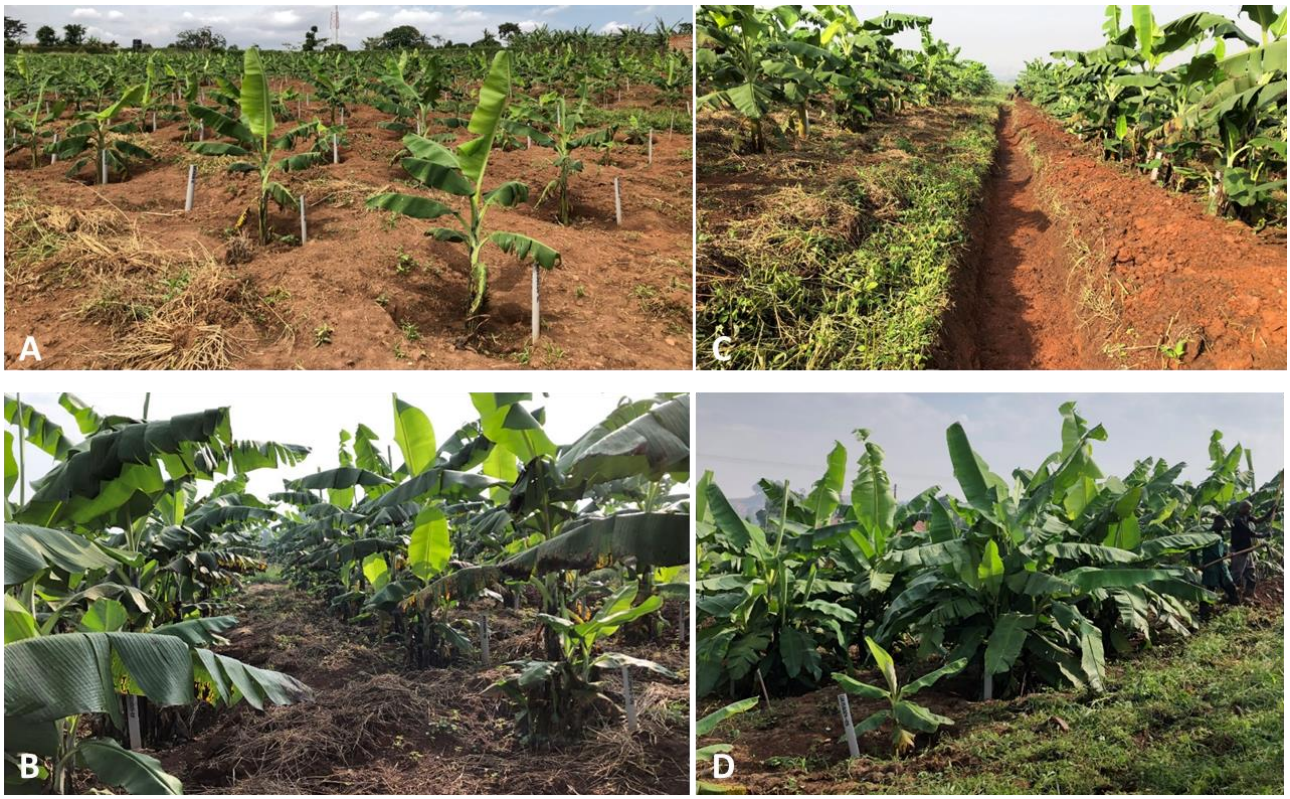


Figure 2:8: Status of the CFT to-date (A) 2 months after planting, (B) Mulching for weed control, (C) Trenching for soil and water management, and (D) Current state – 7 months after planting.

2.1.1.7 Activity 1.1.6: Compliance with NBC regulatory requirements

In fulfilment of regulatory requirements and in accordance with the Standard Operating Procedures (SOPs) and National Guidelines for Field Trials of Genetically Engineered Crops by Uganda National Council for Science and Technology (UNCST), the Project team submitted a Planting Report to the NBC, and conducted a trainings in Biosafety and Risk Assessment and weevil and nematode assessment.

Additionally, both technical and support project staff including laboratory, field and the security personnel underwent a stewardship training in 'Biosafety and Data Collection'. The Project team underwent a training where select NBC members participated as resource persons. All new staff especially the security guards have been taken through the SOPs and guidelines that govern management of CFTs. The team will continuously undertake refresher trainings for existing and new staff.

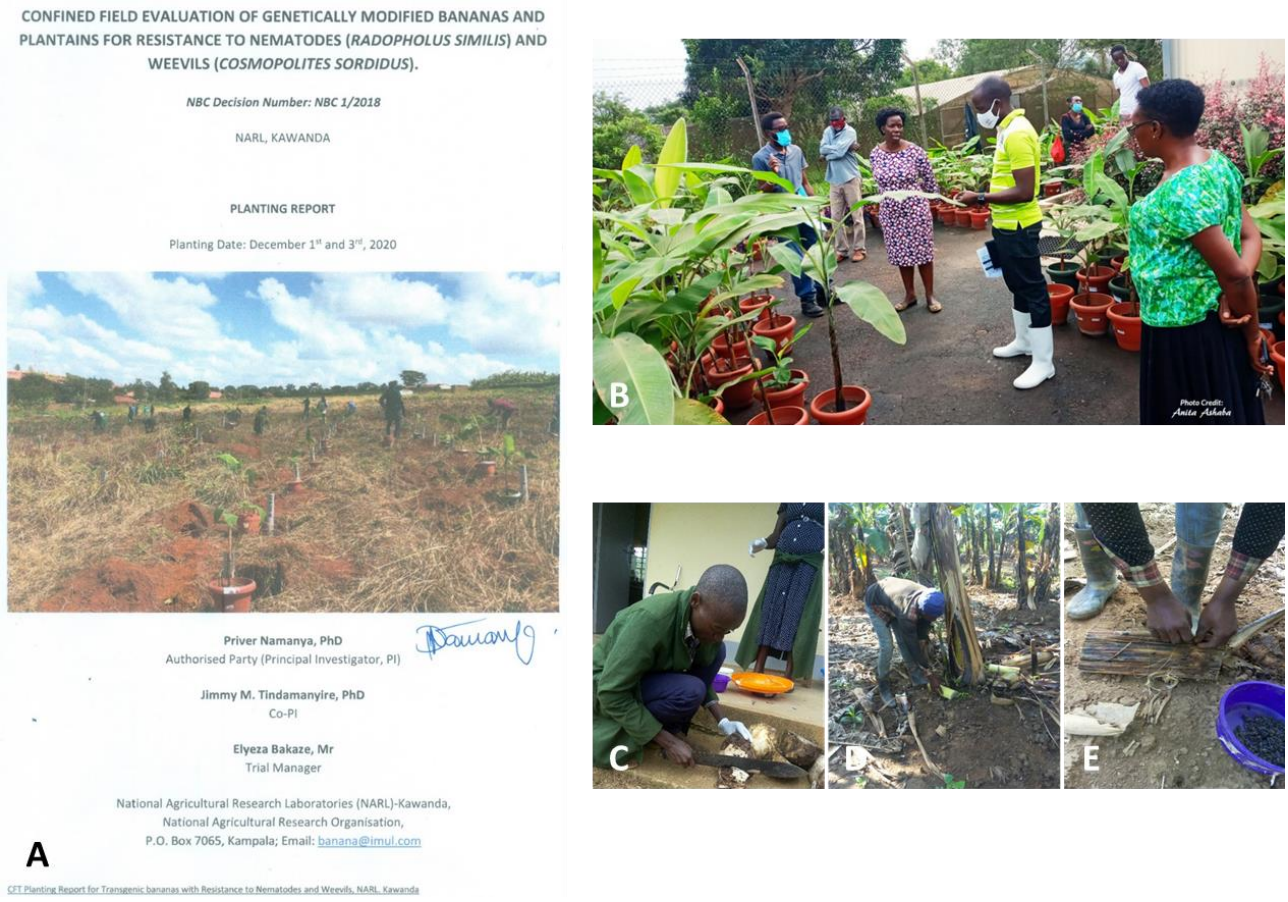


Figure 2:9: Compliance with NBC (A) Planting report submitted to NBC, (B) Pre-planting briefing by MAAIF inspector and (C – E) training project team to rear and assess weevil damage.

2.2 Objective 2: Generate new technologies for Foc and emerging traits

Uganda's dessert banana industry is dominated by Gros Michel and apple banana (Sukali Ndizi) which are both susceptible to Foc race 1 (Foc 1) disease. Studies show Foc 1 resistance in the EAHBs which showed differentially enriched primary metabolic and ribosome pathways. Many genes linked to (i) oxidative burst, (ii) cell wall strengthening, and (iii) antifungal proteins are induced in response to Foc 1 infection. Therefore, the Project will use the available Musa genome sequence to identify and isolate antifungal genes from selected EAHBs to address Foc 1 resistance in dessert banana.

2.2.1 Activity 2.1 Gene isolation and cloning.

Sub-activities include (i) Maintain selected genotypes, establish in tissue culture and wean in pots, (ii) Infection stages - prepare Foc inoculum and inoculate genotypes, (iii) Induce/express target genes in banana cultivars, (iv) Extract total RNA and genomic DNA and (v) Design PCR primers, identify target genes and their safety.

2.2.1.1 Activity 2.1.1: Maintain selected genotypes, establish in tissue culture and wean in pots

Two banana genotypes were selected, multiplied *in vitro* and weaned from which 50 plants were potted in preparation for infection with Foc race to induce expression of target genes (Fig. 2.10).

Figure 2:10: Multiplication of plants; *In vitro* plants (A), weaned plants (B) and plants infected with Foc 1 (C).

2.2.1.2 Activity 2.1.2: Design PCR primers, identify target genes and their safety

Using bioinformatics tools such as AllergenOnline (Home of the FARRP allergen protein database, http://www.allergenonline.org/), the Project analysed, identified and selected 9 target genes to be isolated and applied in offering Foc 1 resistance to dessert bananas. The safety of target genes' proteins at 80mer was confirmed at "No Matches of Greater than 35% Identity Found" using AllergenOnline (Fig. 2.11).

```
The best scores are:                                      opt bits E(2233) %_id  %_sim  alen
gi|123299282|gid|449|allergen Cry j 2 [Cryptomeria j (  65)   65 26.6    0.69 0.308 0.654   52

>>>query, 481 aa vs version2136.fasta library

>>gi|123299282|gid|449|allergen Cry j 2 [Cryptomeria japonica]            (65 aa)
 initn:  40 init1:  40 opt:  65  Z-score: 108.5  bits: 26.6 E(2233): 0.69
Smith-Waterman score: 65; 30.8% identity (65.4% similar) in 52 aa overlap (43-87:11-62)

             10        20        30        40        50            60           70
query  VEALLSSFLEILIDSTKKSVVRQIGAVWGLEEDLEKLGRTLLRIQSIVGDAEEQQ----IKDTAVKKWLTA---LRDAAY
                                          .: .: :.  : :.:    . :..:.:.: .   :: . .
notag|                                MAMKLIAPMAFLAMQLIIMAAAEDQSAQIMLDSVVEKYLRSNRSLRRVEH
                                             10        20        30        40        50

          80        90       100       110       120       130       140       150
query  AAEDVLDEFNLEILRKSNRAIENKMMGKVSDFFSSHNALYFRFKMARKLNEVVKSIDEIAAESRKFNFAVRTQEQTPPTV
       . .:... ::.:
notag| SRHDAINIFNVEKYG
               60
```

Figure 2:11: Output of AllergenOnline bioinformatics tool shows selected target gene with less than 30.8% which shows "No Matches of Greater than 35% Identity Found".

2.2.1.3 Activity 2.1.3: Infection stages - prepare Foc inoculum and inoculate genotypes

Three pure isolates (NdK 01, 02, and 03) of Foc 1 were isolated and confirmed from diseased banana plants collected at NARL and tested for virulence on potted Sukali Ndizi plants (susceptible to Foc 1) (Fig. 2.12). Potted Sukali Ndizi plants showed wilting 14 days after infection with Foc 1 – confirmed its virulence (Fig. 2.10, C).

The Project has developed Protocols and SOPs for inoculation of root systems with Foc 1.

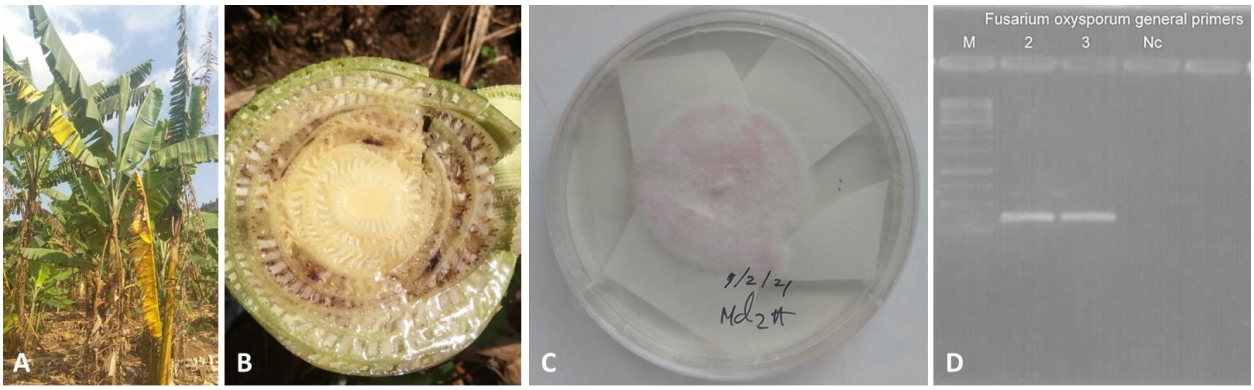


Figure 2:12: Pure isolates of Foc 1; Foc race 1 symptoms in infected banana plant (A – B), Foc race 1 culture on PDA media (C) and Foc race isolates confirmed by PCR (D).

2.2.2 Activity 2.2 Staff skill support – Gene annotation for RNA and DNA sequencing.

The Project recruited of 4 security guards, two field technicians and four field assistants to manage the CFT activities. In addition, 2 research assistants have been recruited for isolation and cloning of Foc 1

resistance genes. This team has undergone orientation trainings in biosafety, risk assessment and stewardship of GMO crops, data collection and management of project activities. However, no skilling of staff in areas of gene annotation for RNA and DNA sequencing has been achieved yet.

2.2.3 Activity 2.3 Tissue culture systems.

The Project was expected to achieve this through a PhD studentship to develop protoplast regeneration system but the student has not been recruited yet.

2.2.4 Activity 2.4 Gene editing.

The Project was expected to achieve this through a PhD studentship to undertake gene editing of the dessert banana genome using CRISPR/cas9 to develop bananas that are resistant to Foc 1. This student has also not been recruited yet.

2.3 Challenges.

This phase of the Project has two objectives, that is, (i) To evaluate and release nematode and weevil resistant banana products and a tracking system and (ii) To generate new technologies for Foc and emerging traits. This report has therefore shows that all Year 1 planned activities of Objective 1 were met while Objective 2 activities were largely hampered mainy due to the limited funds that were transferred to NARO also that largely affected the recruitment of PhD students. Additionally, the emergency of the COVID-19 pandemic also affected which was characterised by total lockdowns made it it difficult to execute project activities making it hard to meet Project milestones.

2.4 Planned Activities for Year 2 (*see Work plan, Section 2.5*).

1. Maintenance of the confined field trials.
2. Maintain weevil populations and post planting weevil infections of CFT.
3. Maintain banana nematode populations for booster inoculation.
4. Agronomic data collection in the CFT.
5. Isolating and cloning of Foc 1 resistance genes;
    a. Conduct banana root infections with pure Foc 1 cultures.
    b. isolate total RNA followed by synthesis of complementary DNA (cDNA)

3. Germplasm collection and its management

The regional Banana Germplasm Collection Centre for the East and Central Africa (ECA), located in Mbarara district, Uganda (Figure 3.11), continue to house the different bananas have been assembled from Uganda, Kenya, Rwanda, Burundi, Tanzania Democratic republic of Congo, South Africa, India, Papaua New Guinea, Brazil, Honduras to capture a wider ecological and plant adaptation regimes. The collection at Mbarara is committed to the conservation of maximum variability of both local and exotic germplasm to enable the sourcing of important key traits to be used in the crop improvement programmes (Figure 3.12). This work is achieved through a) collecting and accessing unique and new germplasm b.) pre-breeding characterization, evaluation and identification of genotypes with maximum potential for resistance against pests, diseases and stresses in the environment as well as good agronomic and yield characteristics, c) dissemination of materials to different stakeholders and providing support to students and researchers through identification and describing of materials being used in the course of their studies.

Figure 3.11: Current state of the regional germplasm at Mbarara

This current Biotech project phase ensure that the germplasm management and conservation activities are conducted well. There is one research assistants and three casual laborers hired to carry out all activities for collection management and conservation. Fertilizer application was conducted in whole germplasm in March 2021.

Figure 3.12: A selection of the germplasm found at Mbarara regional collection Centre

5. Human capacity: PhD studentships.

NARO coordinated students: This phase of the Project has two objectives, that is, (i) To evaluate and release nematode and weevil resistant banana products and a tracking system and (ii) To generate new technologies for Foc and emerging traits. This report has therefore highlighted the progress made thus far in objective (i). The Project has not been able to commence activities in Objective (ii) mainly due to the limited funds that were transferred to NARO which also affected the recruitment of PhD students. Additionally, the emergency of the COVID-19 pandemic has also affected commencement of preliminary activities.

Bioversity International coordinated students: The two Ph.D students who initiated their research during phase three of the project are progressing well. A summary of their project activities is summarized below:

1. **Developing genetic tools for integrating drought tolerance in East African highland banana production systems (Moureen Nasamba)**

Breeding requires prior selection of appropriate male and female parents. Majority of farmers in the East African region, particularly in Uganda prefer EAHBs, which have been used as female parents in current breeding programs. However, most of these EAHBs are very sensitive to drought. Therefore, this study aims to contribute to the improvement of East African Highland bananas through identification and introgression of drought tolerance genes into the EAHB populations. Specifically, screening of germplasm is required to identify tolerant candidates, which can be used as male parents in subsequent crosses to develop F1 population(s), which will then be characterized by phenotyping and the breeding potential of selected candidates established. This study also aims to characterize the developed F1 population by genotyping to identify molecular markers linked to drought tolerance in banana. Putative drought tolerance genes identified in this study can be isolated, cloned and incorporated (individually or stacked) into farmer-preferred but drought sensitive genotypes using biotechnology techniques such as genetic engineering.

The goal of this study is to contribute to the improvement of East African Highland bananas through identification and introgression of drought tolerance genes into the EAHB populations. Below are the specific objectives and the progress for each one of them.

Objective 1: Review breeding for drought tolerance in banana

For this objective, a systematic review of research efforts towards understanding and breeding for drought tolerance in bananas was written and published in the journal, Plant Breeding. The publication is titled *"Breeding banana (Musa spp) for drought tolerance – a review"*.

Objective 2: To assess effects of drought stress on banana production and identify management practices by farmers

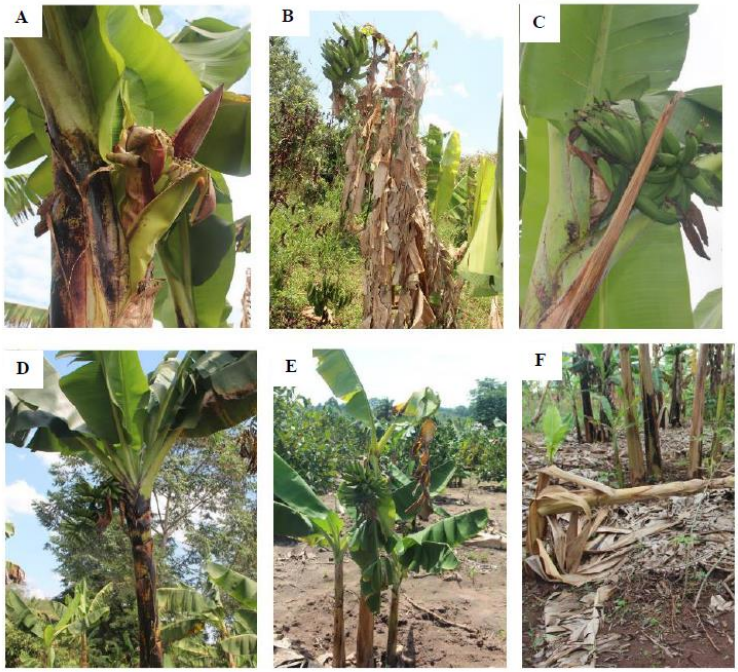
A survey was conducted in banana growing districts in Central and Southwestern Uganda to determine drought stress effects on cultivars grown by farmers and identify on-farm coping practices employed by farmers for mitigating drought effects on bananas. Data collection was completed. Common drought effects on banana production included wilting and drying of leaves, reduced bunch size, stunted growth, strangled birth and reduced number of fingers or clusters. Most popular farmer coping strategies included mulching, planting of mixed cultivars, construction of trenches to trap soil and water, reduced leaf harvesting, application of manure and irrigation. For this objective, data analysis and interpretation is on-going.

Objective 3: To screen banana germplasm for drought tolerance to select candidate genotypes

Thirteen *Musa* genotypes were screened for drought tolerance based on morphological and physiological traits under screenhouse conditions. All *Musa* spp. genotypes showed high susceptibility to water stress. Sensitivity to moisture stress was manifested as significant reductions in total leaf area, total biomass production, plant height, number of leaf cigars and functional leaves, chlorophyll content and stomatal conductance. All genotypes had increased water use efficiency (WUE). 'TMB2x6142' had the least reduction in the total dry matter, total leaf area and highest WUE and hence was the most tolerant to water stress. However, we recommend screening of these genotypes under field conditions to confirm if these observations translate in high yield. This objective's manuscript was submitted for review under the title *"Evaluation of banana (Musa spp.) for performance under drought stress based on phenotypic and physiological trait responses"*.

Objective 4: To generate F1 population(s) and characterize them for drought tolerance

For this objective, crosses will be done to generate an F1 population, which will then be phenotyped for drought tolerance to see if they will segregate for drought tolerance-related morphological traits used in objective 3 above. This F1 progeny will also be genotyped using DART sequencing to identify genetic markers linked to drought tolerance.



Symptoms of drought stress on banana plants: (A) strangled birth (B) wilting and drying of leaves (C) reduced bunch and finger size (D) formation of petiole rosette (E) stunted growth (F) snapping of weak pseudo-stem

2. **Characterization of the effects of banana root exudates on Fusarium oxysporum f.sp cubense race 1 in mixed cultivar systems (Georgina Mwaka)**

Previous studies suggest that banana root exudates break the dormancy of the hard-cased *Fusarium oxysporum* f.sp *cubense (Foc)* spores. However, there is still poor knowledge on *Foc* and the banana root system especially in EAHB. This study, therefore, seeks to determine the effect of mixed cropping on development of *Foc* incidence and severity in the susceptible banana cultivars in Uganda. This study aims to determine the composition of banana root exudates from the resistant EAHB and their effect on *Foc* race 1 spore germination and hyphae development. The study will further determine the differential beneficial microbes between the resistant and susceptible varieties, influenced by mixed cropping and how these microbes affect *Foc*. Understanding the mechanisms through which mixed cropping and banana root exudates may minimize the impact of *Foc* in susceptible varieties, will not only provide strategies for disease management, but will also ensure continuity of these locally accepted *Foc*-susceptible varieties in Uganda. This study is hoped to form a baseline for genetic transformation of Foc-susceptible bananas with the target gene to enhance disease resistance and protection.

Research progress during the reporting period is addressed per objective as follows:

Objective 1: Fusarium wilt survey in Uganda

The field Fusarium wilt survey including looking at disease incidence based on farmer's cropping practices was completed. 100 farms have been surveyed and data is being analyzed statistically and with GIS to determine correlation of different parameters with Fusarium wilt incidence. Two manuscripts are being drafted 'The distribution and incidence of Fusarium wilt of bananas in Uganda based on farmers cropping practices in mixed cultivar systems' and the 'Building towards sustainable management of Banana Fusarium wilt race 1 in East and Central Africa'. PCR confirmation of isolates from the different districts is also going to begin.

Objective 2: The effect of mixed cropping on Banana Fusarium wilt development in susceptible varieties in Uganda

Four on-farm trials that had been set up in Nakaseke and Luweero district looking at the effect of intercropping Foc-resistant and Foc-susceptible varieties on Fusarium wilt incidence and severity is ending. The trials will be terminated late August to September 2021. Preliminary results considering external symptoms show that severity is reduced by 50% but incidence is relative. However, corm ratings and pictures taken will be done at trial termination. Data analysis with ANOVA will be done to determine significant differences between treatments. QPCR will also be done to determine FOC load in the different treatments. The on-station trial will continue much longer to get better data. We will also be setting up some pot trials.

Objective 3: Soil microbe analysis from the trials

At trial termination above, soil and banana roots samples will be collected for soil microbe analysis. Then total DNA extraction will be done and if funds are available, they will be sent for sequencing. At least 20000 USD would be required to get good results. There will be need to purchase more soil DNA kits.

6. **Construction of Tissue Culture laboratory at NARL, Kawanda**

The project continues to focus on infrastructural development and maintenance in the quest to provide a solid basis for new scientific advances. The ever-increasing research activities, equipment and research personnel in tissue culture and biotechnology necessitate more lab space. To meet the demand, the Project is constructing a new Tissue Culture facilitate that will be completed by end of 2020 (Figure 3.13). The lab which is 750 square metres is aimed at contributing to the overall goal of NARL-NARO to bring about positive change in the livelihoods of producers by generating technologies for improving and sustaining productivity of the mandate commodities and systems. The laboratory will specifically contribute be generating over 5 million of tissue culture planting material for both banana and other horticultural crops.

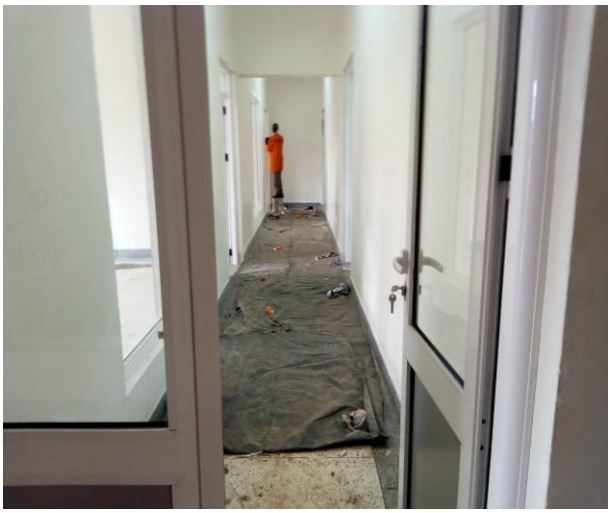
Figure 3.13: Pictorial of the laboratory when complete

The laboratory is currently about 90% complete (Figure 4.14). The external works, installation of ACs and fencing remains to be done which are expected to be complete by end of December 2021. Funds for completion are being mobilized.

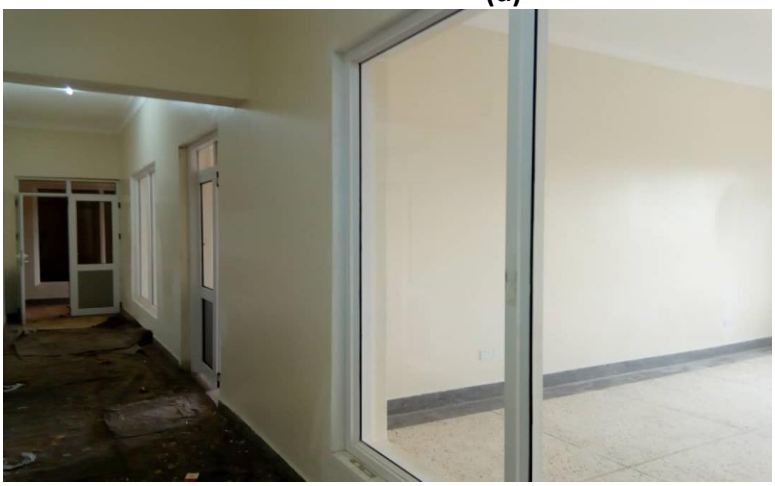
(a)

(b)

(C)



(d)



e)

Figure 3.14: Photographs of the laboratory construction progress: (a) front view, (b) behind view, (c) verandah (d)/(e) Internal view

7. Project management

As has been the practice for this project in the last three phases, a Steering Committee (SC) to oversee the activities of this project in terms of technical guidance for the implementation was selected. The SC first met in January and February 2020 to set priorities of project activities and agree on project budgets. The project project harmoinsed the budgets and has so far prepared two letters of agreements in favour of the project implementing partner, NARL- Kawanda for 2019 and 2020-21. The project has been able to conduct a steering commettee meet on 15 to 16th June 2021. The next project review and SC meeting will be conducted in June 2022.

8. Workplan.

Project workplan	Pre-Implementation				YEAR 1				YEAR 2				YEAR 3				YEAR 4				YEAR 5			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Objective 1: Evaluate and Release nematode and weevil resistant banana products and a tracking system.																								
1.1 Select elite lines from CFT at Kawanda																								
•Establish and maintain plants in glass and screen house facilities																								
•Propose CFT site for NBC approval																								
•CFT Application submitted and NBC approved																								
•Establishment of CFT faciltities at NARL, Kawanda																								
•Inspection and Approval of CFT facilities by NBC																								
•Collect, Establish and maintain Nematode innoculum in potted plants																								
•Collect, Establish and maintain Weevil innoculum in potted plants																								
•Nematode innoculation (Pre-planting)																								
•Planting of CFT at NARL, Kawanda																								
•Weevil innoculation (Post-planting)																								
•Primary molecular characterisation (PCR) - resistant lines																								
•Weevil damage data																								
•Nematodes population build-up /damage data																								
•Agronomic and yield data collection																								
1.2 Detailed molecular analyses to meet regulation requirements.																								
•Southern blots to select lines with 1-3 copies and eliminate duplicate lines																								
•PCR for presence of back-bone sequences.																								
•Expression studies by qPCR and ELISA .																								
•Gene insert analysis to ensure no new ORFs.																								
1.3 Advance elite lines from MTL for commercial release.																								
•In vitro multiplication of 5 selected lines																								
•Establish and maintain 3 CFT sites: Kawanda, Mbarara, Bulindi-Hoima.																								
•Weevil damage data																								
•Nematodes population build-up /damage data																								
•Agronomic and yield data collection																								
•Determine fruit compositional data.																								
•Collect environmental, allergenicity and toxicity data.																								
•Collect data on Distinctness, Uniformity and Stability (DUS).																								
•Consumer sensory acceptability data.																								
•Compiling the Deregulation dossier																								
•Develop a tracking and stewardship system to deliver products of integrity																								
Objective 2: Generate new technologies for Foc and emerging traits.																								
2.1 Gene isolation and cloning.																								
•Maintain selected genotypes; Establish in tissue culture and wean in pots																								
•Infection stages; prepare Foc inoculum, inoculate genotypes																								
•Induce/Express target genes in banana cultivars.																								
•Extract total RNA and genomic DNA.																								
•cDNA synthesis																								
•RNAseq / transcriptome analysis to identify genes up/down regulated.																								
•Design PCR primers, identify target genes and their safety																								
•Cloning, sequencing and analysis of target genes.																								
•Build binary transformation vectors with Foc resistance genes.																								
•Generate transgenic banana plants with Foc resistance genes.																								
•PCR characterisation of putatively transgenic plants																								
•Screen transgenic banana lines for resistance to Foc.																								
2.2 Staff skill support - Gene annotation for RNA and DNA sequencing																								
2.3 Tissue culture systems- Develop protoplas regenratation system -PhD																								
2.4 Gene editing, annnotation for RNA and DNA sequencing -PhD																								
3.1 Management of the Germplasm Collection																								
3.2 Faciliting two PhD students from phase III to complete																								
3.3 Pre-breeding Characterisation and Breeding																								
3.4 Installations/Fittings for the Tissue Culture lab																								
3.5 Steering committee meetings																								
3.6 Planning/Review meetings																								
3.7 Final project Evaluation																								