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SOP06

Standard Operating Procedure (SOP) for Hybridization (Crosses for banana hybrid development)

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Standard Operating Procedure (SOP) for Banana Hybridization

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1. Introduction

The purpose of this document is to describe the SOPs for banana breeding activities and data management. These activities include crossing, evaluation trial establishment, phenotyping, selection in both early evaluation and preliminary yield trials, sensory evaluation, biophysical and biochemical analysis, selection to advanced yield trial, participatory on farm testing using the mother-baby trial approach, variety release procedure and cultivar registration, data analysis, quality checks to ensure germplasm is still true to type, SNP genotyping of new hybrids for future use in GWAS or GEBV for selection and germplasm management.

Botanical characteristics of the banana plant

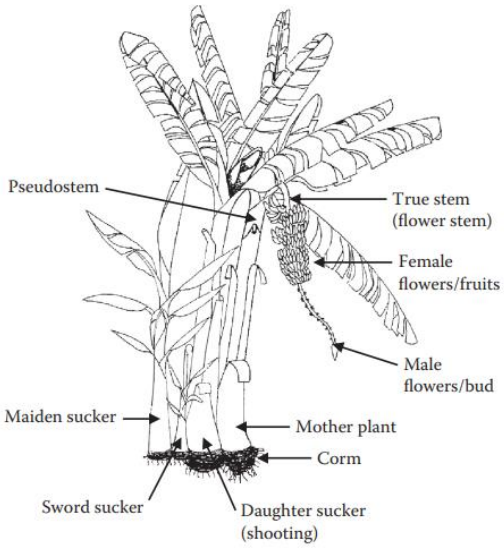

Figure 1. The morphology of the Musa plant: a mat or stool (adopted from Karamura et al. 2011).

2. Purpose

The purpose of this SOP is to describe a series of activities carried out before, during and after pollination/crossing is done when developing hybrids for the conventional improvement of Matooke.

3. Scope

The SOPs apply to all breeding activities implemented under the IITA banana breeding programme in Namulonge, Uganda in collaboration with the National Banana Programme of NARO, Uganda. It aims to reach the broader banana breeding community in Uganda and for use as a blue-print in other banana breeding programmes in Africa.

4. Definition of terms

- **Hybrids:** Varieties of banana plants generated after crossing two different varieties.
- **Checks: Known** Varieties susceptible or resistant to the constraint used as controls in experimental trials.

5. Roles and Responsibilities

All staff involved in implementing breeding activities in the banana breeding programme at IITA-Uganda must use the SOP manual. No alteration should be made to the procedures unless approved exceptionally by the programme leaders. The SOP manual will be revised once a year for possible updates. The list of individuals responsible for each section of the SOP in the breeding activities are listed below.

Senior banana breeder: Responsible for managing and overseeing all trials and data production conducted by the banana breeding programmes of IITA in East Africa

Research associate/Data Curator: Responsible for the creation of trials and all aspects of the trial data once it is established. This includes trial creation, its management and data collection, and checks on the implementation of defined protocols on sites ensuring that meta data for all trials is accurately recorded, has no outliers, is complete and uploaded in Musabase. Provides end-user support and training on the usage of Musabase and digital tools.

Field technicians: Perform field tasks such as such as performing the hand pollination, harvesting, seed extraction, and field data collection or in field management practices as defined in the trial protocols using digital tools defined in the protocol for capturing, storing, transmitting, and ensuring quality of data within defined time periods.

Laboratory technicians: Perform laboratory tasks such as embryo rescue, culturing and subsequent sub-culturing and eventual weaning of plantlets in tissue culture laboratory. They also ensure tissue culture activities are recorded with the ODK App and uploaded on and uploaded to the Banana Tracking Tool (BTracT). Molecular laboratory technicians perform tasks like ploidy analysis for hybrids and DNA extraction for quality control and other breeding related needs.

Data technician: Ensure the quality and accuracy of collected data follows the protocol of each trial. Ensure that collected data are daily backed up, devise and implement efficient and secure procedures for data handling and sharing with the concerned scientific team. Work with the database curators to organize and maintain data in the correct formats for upload and storage on Musabase. Keep good custody and maintenance of data collection gadgets and apps,

identify, justify, and report any need for updating, upgrading, or replacing the data collection gadgets. Assist with reports and data extraction when needed.

6. Procedure/Protocols

6.1 Experimental Planning

6.1.1 Required tools

- Musabase

This is the global banana breeding database and a data management tool in support of breeding. Use this system to generate the crossing plan as stipulated in the breeder's wish list of parents and priority order. It can be accessed at https://musabase.org/.

- Banana Tracking Tool (BTracT)

The banana tracking system for crosses, seeds and plantlets accessed at http://btract.sgn.cornell.edu/btract/.

- Android device
- Plant ID field labels

Product OL125LP - Weatherproof Polyester Laser - 4" x 2" Shipping Labels purchased from 4" x 2" Shipping Labels - Weatherproof Polyester Laser - OL125LP (onlinelabels.com).

- Zebra mobile printer
- Zebra ZQ520 series.
- Zebra desktop printer
- Zebra ZT411 industrial printer.
- Zebra designer software
- Zebra designer Professional version 3.5.
- Cross ID field labels
- SC-TTL - Revolutionary Self-Contained Thermal Transfer Labels. 2.0mil Polyester with a resin ribbon (240 per roll), 3.375" wide, .75" core, 2.25" OD.
- Cross ID lab and nursery labels
  PP White self-adhesive film Plain labels special layout 3 Across 33 x35 mm packed in rolls of 5,000 pcs on 76 mm Core.
- Barcode scanner
- Zebra DS2208-SR Handheld 2D Omnidirectional Barcode Scanner
- Computer with internet access

6.1.2 Parent identification and establishment of crossing plan

The list of parents to be used as males and females, is provided by the breeder together with the crossing plan referred to as 'wish list' here after. We perform triploids (3x) x improved diploids (2x) crosses to create tetraploids (4x) that have desirable attributes of both 3x and 2x parents; however these 4x hybrids are very fertile and contain a lot of seeds. So, we develop secondary triploid progenies that are sterile, parthenocarpic and have additional favourable alleles by crossing the tetraploids with improved diploids. The crosses are also awarded order of priority, with '1' being the highest

priority. This means crossing plan with priority = 1 should be performed first if female plant is ready and pollen is available. The breeder determines priority based on the attributes of the product profile and past performance of the parents. The choice of parents and priority order can be changed if the breeder suggests the need to. An example of this is seen in table 1 below. Follow this link to create a crossing plan. https://solgenomics.github.io/sgn/03_managing_breeding_data/03_06.html#cross-wishlist

Table 1. An example of a crossing plan.

Table 2. An example of a crossing plan.

		Male (all 2x)							
Female	**Ploidy**	10969S-1	5265-1	5610S-1	8075-7	9128-3	CV. rose	Malaccensis_250	SH3217
Entukura	3x		X		X	X	X	X	X
Enzirabahima	3x		X		X	X	X	X	X
Kabucuragye	3x		X		X	X	X	X	X
Kazirakwe	3x		X		X	X	X	X	X
25974S-17	4x	X		X	X		X	X	
29275S-5	4x	X		X	X		X		
29364S-2	4x	X		X	X			X	

Key
Above dotted line: Triploid (3x) female parents
Below dotted line: Tetraploid (4x) female parents

X: Priority 1
X: Priority 2
X: Priority 3
X: Priority 4
X: Priority 5

6.1.3 Adding trial to Musabase

Detailed step-by-step instructions on how to create or add a trial in Musabase are available at: https://solgenomics.github.io/sgn/03_managing_breeding_data/03_07.html#adding-trials

- No experimental design is followed for pollination blocks.
- Create defined lists of accessions to be used as male and female parents.
- Add the lists to the database.
- Use the lists to add the accessions to the database if they have not been added yet.
- Create an excel file in csv format of the field layout.
- Add the trial to Musabase entering all relevant Meta data in “Trial Information” like trial name using naming convention, location, plot and field dimensions.

6.1.4 Field preparation

- Choose land that is relatively flat for efficient cultivation and management.
- Clear the land of overgrown bushes by hand slashing.
- Do a first plough of the land to break hard surface and open it up with a tractor.

- Do a second plough 3-7 days after first plough using a tractor mounted with a harrow disc that deeply cut to loosen up and mix the soil thoroughly.
- Measure and field mark to spacing of 3 metres (between plants) x 3 metres (between rows) for triploid and tetraploid parents, giving a plant density of 1,111 plants/ha.
- Measure and field mark to spacing of 2 metres (between plants) x 3 metres (between rows) for diploid parents, giving a plant density of 1,666 plants/ha.
- Dig or drill planting holes measuring 60 cm (2ft) wide and 60 cm (2ft) deep.
- Separate the topsoil from the bottom soil while digging the planting holes to be used at planting.

6.1.5 Preparation of planting material

- If using tissue culture plants, choose healthy and strong looking plants with a girth of 5.0 cm and above for planting. We use girth and not height because we need plants that will not break during transportation and planting exercises.
- If using suckers, pick the sword suckers and pare the corm by removing several thin layers and discoloured tissue to reduce nematode infestation.
- Dip the pared corms in boiling water (100° C) for 30 seconds to kill any other pathogens left on the surface.
- Alternatively dip the pared suckers in pesticide solution containing 50 ml of Dursban (active ingredient 75 % chlorpyrifos) in 20 l of water.

6.2 Planting crossing blocks

- Planting is done at the start of the rain seasons (March-April for season one and August-September for season two).
- Mix earlier separated topsoil with 20 kg of well dried farmyard manure (cow dung is commonly used).
- Place the tissue culture plantlet or pared and treated corm in the middle of the planting hole and add the soil-manure mix until plantlet is firm, or corm is well covered with at least 15 cm of soil.
- Place mulch around the plant (ring mulching) to conserve soil moisture and control weeds. Be careful that the mulch does not touch the plant.

6.3 Creating and printing field labels

Detailed step-by-stop process of how to create and print bar codes is available at http://solgenomics.github.io/sgn/03_managing_breeding_data/03_12.html. The general steps are:

- Open the manage tool on Musabase.
- Click on label designer.
- Select the trial you want to create labels for.
- Create a new design or load a saved design. The saved design for field labels used at Sendusu is named ‘‘Sendusu labels’’ in the database. This design has been customized to fit the bar code label papers mentioned in section 3.5.1.4 above and are as seen in the figure 2 below.

- Download the pdf file of all barcodes of the trial.
- Print the bar codes using a laser jet office desktop printer. The saved design ‘Sendusu labels’ mentioned above is customized to print one bar code label per plot.
- Stick the bar codes on 0.5-metre-long PVC pieces, flattened at the area of bar code placement.
- Label the plants by placing the labelled PVC pieces in front of the plant

Figure 2. Showing an example of the Sendusu label design for bar codes.

6.4 Field maintenance

- Mulch the fields with dry grass to a thickness of about 10 cm to control weeds and conserve moisture.
- Control weeds by manual removal of weeds using hand hoes as frequent as possible or by herbicides (50 ml of 2, 4-D amine salt 720g/l and 150ml of Agrosate - 480g/L glyphosate, mixed in 15 litres of water) using a Knapsack sprayer If using herbicide, wear a protective gear of waterproof suit, gumboots, gloves, and nose masks to protective yourself against the harmful effects of the herbicide.
- De-trash the fields by cutting off the broken, dead, and dry leaves to keep the fields clean and reduce sigatoka disease inoculum.
- Routinely scout for banana Xanthomonas wilt (BXW) infected plants and destroy them by cutting, collecting them in one place and burning them. Disinfect all tools used with bleach to prevent spread.
- De-sucker the female pollination blocks to maintain three plants, mother, daughter, and granddaughter plants (MDG) per mat regularly
- Cover male buds of the pollen source plants as soon as they come out, with a cotton mesh bag to prevent pollen contamination as seen in the figure 3 below.
- Remove the male buds from female parents after the last cluster has formed to control banana Xanthomas wilt (BXW).
- Always make sure the plant ID labels are well placed on the right plants and replace any damaged bar codes.

a

b

Figure 3. Male bud to be used as pollen source (a) and male bud covered with mesh bag to prevent contamination by foreign pollen (b).

6.5 Using BTracT for data recording

6.5.1 Setting up ODK data collection form

- This is done as soon as the wish list and pollination blocks field layouts have been uploaded and synchronized in MusaBase.
- Export the earlier created crossing plan from Musabase to the ONA server.
- This is done on Musabase via link https://musabase.org/breeders/odk
- Choose an ODK form appropriate for the breeding programme, for example 'BTracTSendusu' for the breeding programme in Namulonge as seen in figure 4 below.
- Import the form and this will make the crossing plan available on the device to be used.

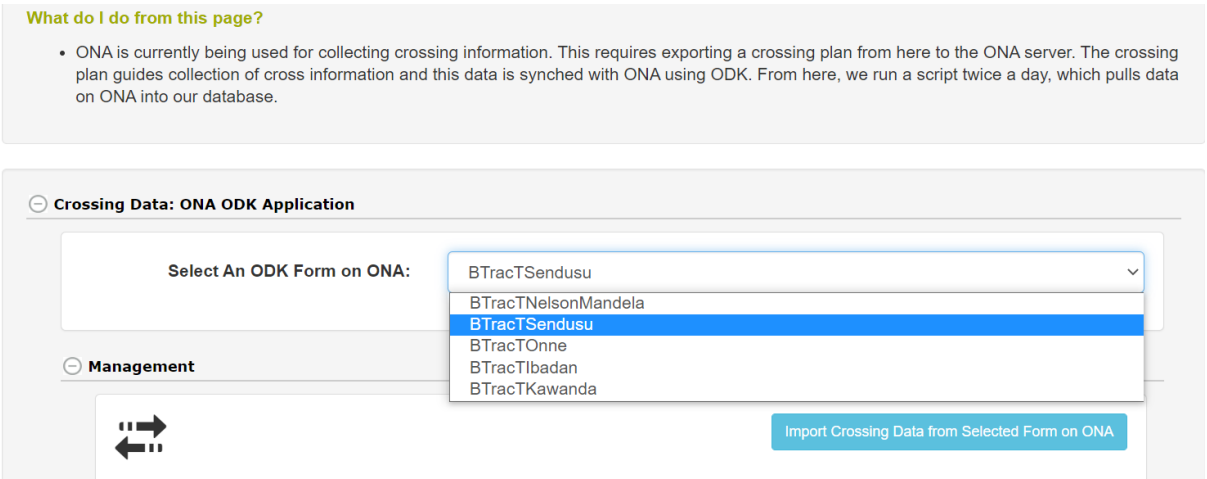

Figure 4. Example of ODK forms available on Musabase.

6.5.2 Setting up BTracT and data recording

- This is done for field and nursery activities and requires internet connection.
- Download and install ODK collect, ODK Sensors Framework and ODK print drivers from the play store in the Android tablet. BTracT operates using these three softwares.

- Detailed step-by-step process of device and account set up can be accessed at http://btract.sgn.cornell.edu/btract/_w_357fae98de45f75473effcfb22e6f879646238197da00b2f/docs/usingbtract.html .
- Open main menu and click '**Get Blank Form**' to load data collection form on the device as highlighted in figure 5
- Select the form to be used for the specific site e.g. BTracTSendusu for Sendusu site.
- Go back to main menu and select '**Fill Blank Form**' as seen in figure 5.
- Select the form displayed, for this case 'BTracTSendusu' to start recording information.

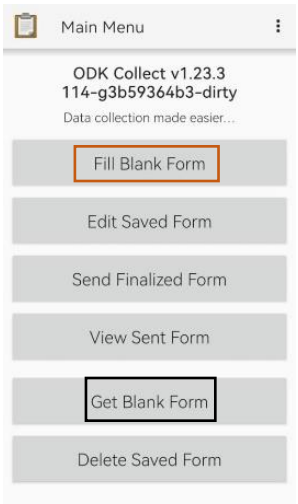

Figure 5. Screenshot of main menu of ODK collect App on a device.

6.6 Crossing

- Scout for flowering female plants and record them. This is done daily in the evening of the day before pollination or day of pollination.
- Collect pollen as early as 06:00 - 06.30 am from male parents and place the pollen from each genotype in its own polyethene bag. Pollen from different plants of the same genotype can be placed in the same bag.
- Scan the field label of the pollen source plant (male plant) and reprint the barcode label on the mobile printer. See process in figure 6.
- Place the reprinted bar code on the bag containing the pollen as seen in figure 6.

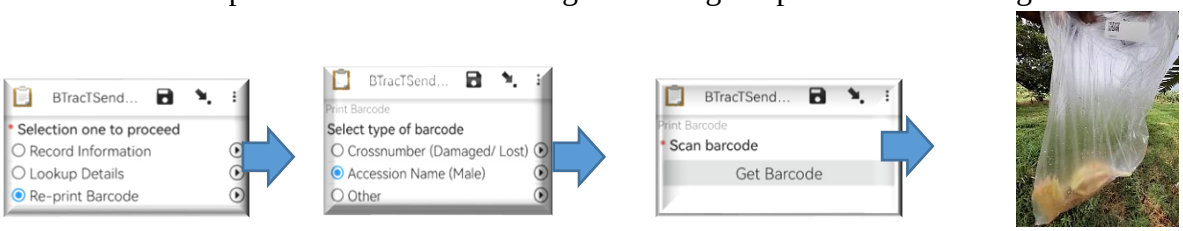

Figure 6. Process of labelling pollen collected for pollination, with a barcode.

- Find the female flowering plants from the previous scouting records in the first bullet of this section
- Open field activity 'first pollination' on ODK collect app.
- Scan the female plant barcode to be pollinated.
- Select the male genotype according to the pollen available (earlier collected) and order of priority stipulated in the cross plan.
- Scan the male plant barcode of the genotype chosen above (barcode on polyethene bag).

- Pollinate the female plant by rubbing the pollen collected gently over the female flowers with open bracts as early as possible in the morning between 7:00 and 10.30 am because pollen viability reduces with time (Swennen and Vuylsteke, 1993).
- Spray the pollinated flower buds with glucose solution made by dissolving 30 g of glucose in 1 litre of water.
- Proceed to print an automatically generated cross ID barcode and label the cross made.
- Cover the pollinated flower bud to avoid contamination from foreign pollen.
- Continue to pollinate the plant as bracts open until all flowers are pollinated in a process referred to as 'repeat pollination'. To record data for repeat pollination do as follows:
    - Under field activities on ODK collect app, open 'repeat pollination'.
    - Scan the cross ID previously generated in first pollination.
    - Male genotype to use will then be displayed, as you must use the same pollen as during the first pollination.
    - Scan the male genotype displayed as pollen source.
    - Select and record the date of repeat pollination (should be the day of repeat pollination).
    - Save and exit to save records.
    - Cover the bud every time with the mesh bunch cover bag until all pollinations on the plant are done.


a

b

c

Figure 7: a female flower ready for pollination (a), hand pollination (b) and completely pollinated and covered bud/bunch (c).

6.7 Harvesting

- Harvest the bunch when one of the fingers starts to ripen.
- Harvest by cutting the pseudo stem and gently lower the bunch towards the ground to avoid damaging the fingers.
- Cut the bunch off the main plant at about 10 cm of the rachis.
- Use BTracT to record date of harvest by scanning the harvested bunch (cross ID) barcode.
- Finalize the harvest by acknowledging that the bunch has been taken to the ripening chamber, using BTracT.

6.7.1 Ripening

- Place the harvested bunch in a basin or bucket.
- Re-print the cross-ID barcode label of the bunch and place it on the basin or bucket containing the bunch to avoid mix-ups.
- Place the bunch in a dark room with no ventilation referred to as a ripening chamber until bunches are fully ripe as seen in figure 8
- Check bunches daily to just about full ripening and not rotting.

6.7.2 Seed extraction

- Collect ripe bunches from the ripening chamber.
- Pluck off the fingers from the bunch.
- Peel one finger at a time while squeezing through the soft pulp to feel for seeds until bunch is completed.
- Place all seeds extracted in a soft mesh bag/ cheese cloth.
- Wash the seeds with clean running water to remove all the pulp.
- Air dry the seeds.
- Go to BTracT activity of 'seed extraction', scan the barcode of extracted bunch and record all necessary information such as date of extraction, total number of seeds extracted and where seeds will be taken.
- If many seeds have been extracted, utmost 50 seeds should be sent for embryo rescue and the rest taken to the nursery for early germination unless otherwise stated for specific needs.
- For seeds to be sent for embryo rescue, reprint the cross ID and label the envelopes containing the seeds as seen in figure 8.
- Keep seeds awaiting embryo rescue in dry storage shelves at room temperature for a maximum of one week only.

Figure 8. Brief demonstration of banana ripening and seed extraction.

6.8 Tissue culture laboratory activities

Other standard operating procedures for the processes of embryo rescue, germination, subculture, rooting, are followed in the laboratory.

- Record data using BTracT for the laboratory's activities following the questionnaire like prompts as seen in figure 9.
- This web form is available at https://ona.io/home/
- The detailed systematic process for how to record each activity is described in detail at http://btract.sgn.cornell.edu/btract/_w_357fae98de45f75473effcfb22e6f879646238197da00b2f/docs/usingbtract.html

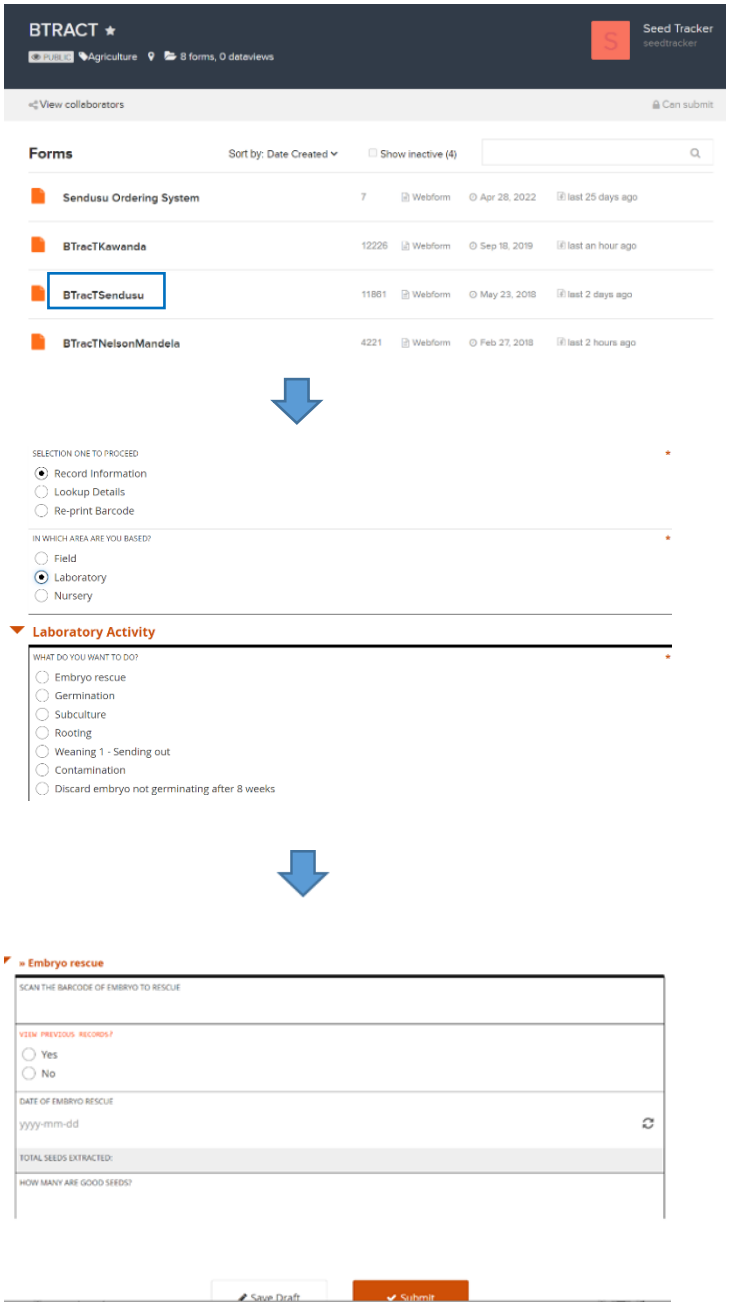

Figure 9. Process of recording data for laboratory activities on ONA.

6.9 Data synchronizing to server

- This process requires internet connection to synchronize activities that were recorded without internet connection.
- It ensures recorded data is exported to the server for cloud storage and access.
- At the end of the field activities for the day and finalizing and saving forms, go to the main menu.
- Click on 'Send Finalized Form' (Figure 10 a)
- Select all forms that need to be sent and click 'Send selected' (Figure 10 b).
- Successful data upload is confirmed with a pop-up message as seen in figure 10 c.

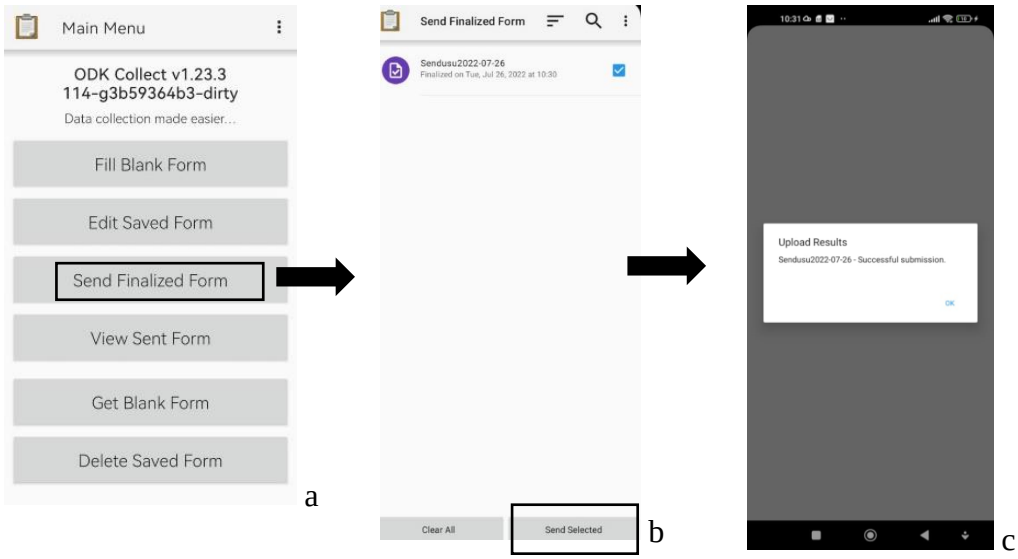

Figure 10. Data synchronizing process on ODK main menu highlighting how to send a form (fig 10 a), select a form for submission (fig 10 b) and successful upload message (Fig 10 c).

7. References

Karamura, D., Karamura, E., & Blomme, G. (2011). General plant morphology of Musa. *Banana breeding: progress and challenges*. 2nd ed. CRC Press, Boca Raton, FL.

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8. Appendix

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