FEDERAL REPUBLIC OF NIGERIA

Application Form for a Confined Field Trial

This form should be forwarded to the **Director-General/Chief Executive Officer, National Biosafety Management Agency** on completion.
This application form consists of seven parts:

1. Administrative information
2. Plant information
3. Trial Description
4. Genetic Confinement
5. Material Confinements
6. Records, Personnel, and Planning
7. Declaration

1. Administrative information

Purpose of Application:
[Application for a confined field trial for (name of Crop species and introduced trait).]
Application for a confined field trial for Cassava (*Manihot esculenta* Crantz).

The aim of the production of these transgenic lines (AMY3 RNAi lines) is to reduce starch breakdown in storage roots of cassava after pruning the shoots, prior to harvest of the crop. The objective is to obtain storage roots with lower post-harvest physiological degradation after harvest (thanks to the pruning) without any loss of the nutritious starch (thanks to the transgene that is hindering the activity of the enzyme catalyzing starch hydrolysis). The construct developed at ETH Zurich in the laboratory of Prof. Samuel C. Zeeman expresses an RNA hairpin homologous to the first 210 base pairs of the Manes.05G097100 gene (Phytozome) encoding a α-amylase targeting it for transcriptional gene silencing. This hairpin is driven by the *Solanum tuberosum* patatin promoter that confines silencing to storage tissues (root-specific promoter). In these organs, starch breakdown will be reduced.

Previous Applications or Approvals:
[Information on the status of this crop and trait, including pending, approved, or denied applications for field trials and commercial releases here or in other jurisdictions. Indicate also if this is a new application or a renewal.]

This is a first-time application by the joint collaboration of IITA - ETHZ Plant Biotechnology Lab to the Nigerian regulatory authorities for a CFT to test transgenic cassava events developed by ETHZ Plant Biotechnology Lab. This is the first application for field-testing of the AMY3 RNAi transgenic lines. They have been previously tested only in screen house at ETH Zurich.
No application has been previously made for the CFT and commercial release of these transgenic cassava clones before in any jurisdiction.

Applicant:
[Name of applying institution, which may also include the name of the Principal Investigator or other key personnel.]

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Proposed Location and Size of Trial:
[Name, address, e-mail, phone, and facsimile of the Trial Manager as well as GPS information or description of the exact location and size of the trial site (attach sketch map).]

Trial Manager: is yet to be employed. A PhD-holder scientist with experience in biotechnology and biosafety will be hired as trial manager upon approval of the CFT. The working place of the trial manager will be at IITA-headquarters, Ibadan, Nigeria in the same campus where the CFT is located. In the meantime, the lead scientist will act as trial manager.
Location of the CFT: IITA-Headquarters, PMB 5320,
Oyo Road, Ibadan 200001, Oyo State
Geographical coordinates of the confined field: 7.490940 N - 3.903835 E
See Annex A for an aerial picture of the CFT site.
Size: The confined field site is about 2900 square meters (31 x 93 m). However, the trial will be conducted on a smaller area (12 x 12 m) within a screen house (see Annex A).

Proposed Duration of Trial: 12 months

Expected starting date: May, 2017

Expected termination date: End April, 2018

2. Plant Information
2.1 Unmodified Plant Information

This section describes the characteristics of the unmodified plant as it relates to confinement. Important information pertains to the plant's reproductive mechanisms and its ability to escape, establish, and persist in the environment into which it is being introduced.

Plant Species Name (Common and Scientific):

Cassava (*Manihot esculenta* Crantz)

Center of Origin:
[What is the center of origin of the unmodified plant?]

Cassava is originally from South America. The unmodified cassava cultivar 60444 included in this study has been selected in IITA from West-African landraces.

Reproductive Mechanism of the Plant:
[Describe the reproductive biology of the plant. This information may be obtained from Organization for Economic Co-operation and Development (OECD), biology consensus documents or similar sources, and should include relevant information on: inter – and intra-specific breeding; pollen production, dispersal and viability; seed production and dispersal; see dormancy, capacity for vegetative reproduction.]

Cassava is monoecious and bears separate male and female flowers on the same plant.
Male and female flowers are borne on the same branched panicle, with female flowers at the base and male flowers toward the tip. In a given inflorescence, female flowers open from one to a few weeks earlier than the male flowers. By the time male flowers open, the female flowers on the same branch have been fertilized or have aborted. However, because flowering on a single plant may last for more than 2 months, pollen from a flower may fertilize other flowers on the same plant, or flowers on surrounding plants, with the proportion of each dependent on the genotype, the environment, and the presence of pollinating insects.
The pollen grains of cassava are relatively large in size, and are sticky. Therefore, wind pollination appears to be of little consequence whereas several species of honeybees (*Apis mellifera*) are considered the main pollinators in Africa. Cassava pollen loses viability rapidly after it is shed.
Pollen viability seems to decline substantially after this time. In practice, breeders take care to perform pollinations within 1 h after collection of pollen to help ensure successful fertilization.
Cassava seeds develop within small fruits, usually three per fruit, with 1-3 seeds being fertile (Jennings and Iglesias, 2002). Developing seeds are viable 2 months after pollination, and the fruit becomes mature about 1 month after that, or about 3 months after pollination. Fruit dehiscence is explosive; the seed initially falls close to the mother plant but then may be further dispersed by ants. Newly harvested seeds exhibit physiological dormancy and require 3 to 6 months of storage at ambient temperature before they will germinate. Seed germination is favored by dry heat and complete darkness.
To conserve the positive attributes of known genotypes, cassava is normally vegetatively propagated by means of stem cuttings, which are known horticulturally as 'stakes'. Stakes are typically at least 20 cm long, and have 4 to 5 nodes each with a viable bud.

Tendency and Weediness:
[Is the unmodified plant regarded by agricultural experts as a weed in regions where it is cultivated? If so, are control methods available that may be used to effectively limit the dispersal and establishment of the unmodified plants? NOTE: The information on the confined field trial location and how the genetically modified plant will be managed are described elsewhere in this application.]

No, cassava is not invasive or weedy.

Toxicity and Allergenicity:
[Is the plant species known to be a source of substances that are toxic or allergenic to humans or animals? If yes, identify the substances and levels that induce toxicity or allergenicity and the affected species.]

Cassava is one of 3,000 plant species that produce cyanogenic compounds that upon breakdown release hydrogen cyanide (HCN) and can therefore be toxic to humans (McMahon et al., 1995).
Cyanogenic glucosides in cassava are linamarin (>90%) and lostraulin (<10%) and cassava varieties have been classified into various groups based on the content of the cyanogenic glucosides, measured in HCN equivalents, or tastes of their roots (McMahon et al. 1995). In the cassava breeding program at the IITA, 100 mg HCN eq Kg-1 is used as the upper limit for cassava classified as low in cyanogenic glucoside content. The cultivars to be field-tested (cv.60444) averages up to 110 mg HCN eq kg-1 and therefore its roots are classified as "slightly bitter" and it requires processing to remove the cyanogens prior to consumption. Such practices are common in Nigeria where household and village-level commercial processing removes the risk of cyanide poisoning and increases the shelf life of cassava-based food products. However, no cassava plant from the experimental field trial will be consumed. These will be incinerated within the CFT site.

2.2 Modified Plant Information

This section is intended to provide information on known or intended effects of the genetic modification or introduced trait that may effect confinement measures employed in the confined trial.

Describe the Intended Phenotypic Changes to the Plant

The intended phenotypic change is reduction of starch breakdown in the storage roots.
The aim of the production of these transgenic lines is to reduce starch breakdown in storage roots of cassava after pruning the shoots, prior to harvest of the crop. The objective is to obtain storage roots with lower post-harvest physiological degradation after harvest (thanks to the pruning) without any loss of the nutritious starch (thanks to the transgene).

Intended Reproductive Effects:

[Does the genetic modification intentionally alter the reproductive biology of the plant? How do these changes affect strategies for confinement?

The genetic modification may result in slower regrowth from stem cuttings (stakes), compared to the wild type. The genetic modification does not intend to alter the reproductive biology of the plants; therefore, it does not interfere with the strategies for confinement.

What is the source of the genetic material? Is the source of the genetic material likely to affect the safe conduct of a confined field trial? If yes, how?

[Describe any known or intended introduction of infectious agents, plant, animal or human pathogens or allergens or toxins.]

The cassava cultivar 60444 originating from Nigeria was used for genetic transformation.

The genetic materials exogenous to 60444 are derived from:
i) Partial DNA sequence from *Solanum tuberosum*; a class I patatin promoter (position 6-999 bp of the Genbank GQ352473.1)
ii) Partial DNA sequence from *Manihot esculenta*; Manes.05G097100 gene (position 1 to 210 of the Phytozome Manes.05G097100 coding sequence)
iii) Synthethic plant intron sequence (57-165 bp of the Genbank M27939 sequence)

The source of the transgenic material will not affect the safe conduct of a confined field trial.

Please, refer to Annex B for further details regarding genetic elements within the integrated vector plasmids and the AMY3 RNAi transgenic lines characterization.

Changes in Toxicity or Plant Composition:

[Describe any changes or toxicity, allergenicity, or significant changes in composition intended by the genetic modification.]

There are no expected changes in toxicity or allergenicity of transgenic cassava clones.

Describe the Features of the Genetic Construct:
[Include coding sequences, promoters, enhancers, termination and polydenylation signal sequences. Attach a genetic map and describe the method of modification in an annex.]

The transformation vector was developed with pCAMBIA1301 as the base vector (GenBank accession AF234297). pCAMBIA1301 is a binary plant transformation vector for use with *Agrobacterium tumefaciens*.
In addition to the components of the hairpin construct described below, it contains:
the hygromycin phosphotransferase II (hptII) plant selectable marker gene from *Escherichia coli* fused to the Nos promoter from *A. tumefaciens* and to the Cauliflower Mosaic Virus (CaMV) 3'UTR polyadenylation sequence.
The transformation plasmid vectors used to create the transgenic cassava lines are referred to as pCAMBIA1301_patatin AMY3 RNAi.
Detailed descriptions of these vectors and the genetic elements as well as a description of the method of modification are presented in Annex B.

The vector was used to insert into cassava:
• The hptII gene (selectable marker) expressing an enzyme that confers resistance to the antibiotic hygromycin B.
• Inverted repeat sequences coding for hairpin double-stranded RNAs homologous to the first 210 base pairs of Manes.05G097100 sequence (encoding a α-amylase). The structure of this transcribed RNA renders it untranslatable, therefore no exogenous proteins are produced.

3. Trial Description

This section describes the purpose of the field trial, the experimental design and data to be collected, including anticipated pesticides use. Include a description of the habitat at the site, and any organisms of conservation concern that may be in the general area.

Trial Description:

The purpose of the field trial is to evaluate the effect of reduced α-amylase activity in the storage roots of the transgenic cassava under a local field condition in Nigeria to confirm results obtained in greenhouses at ETH Zurich.
The field trial will be conducted within the confined field, under a screen-house (see annex A) to reduce the risk of infestation of *Bemisia tabaci*, the whitefly vectoring cassava mosaic disease (CMD), to which the parental cv60444 cultivar is susceptible. Plant leaves will be weekly sprayed with insecticides, alternating *Imidacloprid* and *Lambda-cyhalothrin* (active principles) every 4 weeks.

Ten transgenic lines and the wild-type genotype will be evaluated (see annex B for genetic characterization of the AMY3 RNAi lines). Lines to be tested will be planted in a randomized pattern within the screen-house, 10 plants per line will be analyzed. Plants on the edges of the screen-house will be excluded. Disease-free plants will be produced in vitro at ETH Zürich and shipped by courier to IITA, Ibadan in Nigeria.

Data will be collected at harvest, 10-30 days after pruning, and 20-40 days after stick re-planting.

At harvest, which will be 5 months after planting, the following data will be collected:
1) Plant height
2) Shoot weight
3) Storage root weight
Storage roots and stems will be sampled during data collection in order to determine starch content. Half of the plants (5 per line) will be pruned and the roots left in soil for 10 days. The stem of the harvested plants will be cut and replanted (10 sticks per lines).

Following pruning, the following data will be collected on a daily basis:
1) Scoring of the shoot regrowth
2) Length of the growing shoot
When a suitable stage of regrowth is obtained (est. 10-30 days), storage roots will be sampled in order to determine fresh weight, starch content and to score the post-physiological degradation.

Following stick planting, the following data will be collected:
1) Scoring of the shoot regrowth
2) Length of the growing shoot
When a suitable stage of regrowth is obtained (est. 20-40 days), stems will be sampled during data collection in order to determine starch content.

The trial site is in the forest agro-ecology of Nigeria (see Annex C). The site was cleared of the native forest several decades ago. Therefore, there is no known organism of conservation concern at the test site. Pockets of the original forest that exist in the area are well separated from the test site.

4. Genetic Confinement

This section describes the measures to be taken to ensure confinement of the genetically modified plants and genes. It is based on knowledge of the unmodified crop and the intended genetic modification.

Provide a map showing the location of the trial site, surrounding fields and relevant geographic features such as streams or waterways.

The trial site is located at the IITA headquarters station in Ibadan, capital of Oyo State in South-Western Nigeria. An isolation distance of 100 m will be maintained between the CFT site and any plants capable of hybridizing with cassava. See Annex A for an aerial picture of the confined field trial site, and Annex C for an aerial picture of the location of the trial site, surrounding fields and other geographical features.

Are there wild plant species in the vicinity of the trial site that could be fertilized by pollen from the trial plants, resulting to viable seeds?

There are no plants of wild species near the confined field that could be fertilized by cassava pollen. The only wild Manihot species in Nigeria is *M. glaziovii* (Bock 1984), which is a non-indigenous, ornamental tree species with no weedy characteristics. Few *M. glaziovii* plants are kept in the IITA collection within the IITA campus. However, they are located more than 1000 m away from the CF site and therefore are not at risk to be pollinated by trial plants

Describe mechanisms in place to prevent pollen-mediated gene flow from the plants in the trial site:
[Genetic confinement or reproductive isolation measures are based on the biology of the unmodified plant and the introduced genetic modification, and include isolation distance and /or other measures as justified by the reproductive biology of the unmodified plants, and any intended effects of the introduced traits on their reproductive biology.]

An isolation distance of at least 100 m will be maintained, between the CFT site and any other cassava fields in accordance with the standard for separation used in cassava breeding programs (Kawano et al., 1978). This isolation distance is calculated starting from the outermost border row. The isolation zone will be regularly monitored during and after the trial to ensure the continued absence of any cassava.
In addition, pollen dispersal will be prevented by removal of any male or female flowers during the entire duration of the experiment. In cassava, inflorescence production is preceded by three-way branching of the main stem, making it easy to detect early stages of the flowering process. Weekly monitoring for initiation of inflorescence will take place starting from 2 months after planting and any inflorescence will be removed and destroyed before maturation. Therefore, there will be no possibility for pollen production or distribution.
Furthermore, inside the fenced area, the experimental plants will be surrounded by a 2 meter wide set of guard rows. These will be made of wild type cassava plants (cv. 60444 and other cassava lines flowering at the same time) that will be treated as pollen trap rows. All plants within the cassava guard rows will be incinerated along with the test plants at the end of the field test.
No effects of the introduced traits is intended or expected on the reproductive biology of the test plants.

Describe measures in place to control trial plant volunteers after termination of the trial:
[Describe the crops to be allowed following the confined trial, duration of monitoring or volunteers, frequency of monitoring, methods of destruction and disposal of any identified volunteers, and any other measures needed to ensure that the trial plants do not persist on the trial site.]

The trial spot area will be left fallow and kept free of volunteer cassava plants for at least one year after the conclusion of the trial or until a new trial will be started. The presence of volunteers will be monitored on a monthly basis for a period of six months and volunteer plants found will be uprooted, left to air dry, placed in the incinerator and burnt.

5. Material Confinement

This section describes the mechanisms by which trial personnel will maintain control of the genetically modified plant material, so that it is not mixed with non-modified plant material, does not escape into the environment, and is not eaten by humans or livestock.

Packaging:

[Describe how the genetically modified plant material will be packaged and labeled for transport to the trial site and measures for cleaning and/or disposing of the packaging material. Note that the chain of custody documentation is required for all genetically modified material being transported.]

The experimental plants will be imported from ETH Zurich, Switzerland. Individual plants will be transported in 50 ml clear plastic sealed culture tubes singularly labeled with appropriate identifier unique to this GMO. The tubes will be encased in Styrofoam packaging enclosed in plastic bags and placed in carton boxes. These boxes will be shipped by express courier from Switzerland to IITA headquarters, Ibadan where they will be received by the IITA principal investigator and transported to the biosafety level 2 (BL2) containment screen house facility. After post-entry inspection and clearance by the regulators, plants will be acclimatized and hardened for 4-6 weeks in sterile soil in individual 15 cm pots. After hardening the plantlets in the pots, they will be packaged in closed cartons lined with plastic film to prevent the spillage of any material. These cartons will be transported within enclosed vehicles from the screen house to the fenced field trial site within IITA headquarters under the supervision of a representative of IITA Institutional Biosafety Committee (IBC) and the IITA principal investigator.

The packaging, including the plastic tubes, the Styrofoam boxes, plastic bags, cartons, pots, will be destroyed by burning with a flammable liquid in the incinerator within the CFT site.

A compliance form, material transfer form, and import permit will be prepared and maintained to document movement of the plants to the IITA headquarters containment facility from ETH Zürich, Switzerland and within IITA from the containment facility to the CFT site.

Harvesting, Transport and Storage:

[Describe how the plant material will be harvested, including plans for any material to be retained, and how that material will be stored and/or transported.]

Destructive data collection and harvesting will be performed in the field. Samples will be stored in sealed plastic bags and transported to the IITA BL2 laboratory, Ibadan for further analysis using established protocols, when and if required.

Disposal and Clean-up:

[Describe how surplus planting material will be disposed of at the trial site, how any equipment used during plating or other farm operations will be cleaned, and how harvested materials and crop residues will be disposed.]

Surplus planting material will be retained within the BL2 screen house at IITA headquarters, Ibadan. These plants will be a source of replacement of plants in case of failure of plant establishment in the CFT at IITA thereby ensuring proper field experimentation.

All plant material will be harvested by hand. Cassava sticks of wild-type and transgenic plants will be collected for analysis and replanted in the field. Once plant sampling is completed and data collected, all plants will be dug up, chopped up and allowed to air dry in the sun for 2-3 days after which they will be destroyed by incineration in the existing pit, within the fenced CFT site at IITA, prepared for this purpose. Disposal of all materials will be recorded in the compliance binder. All tools used in the harvest will be washed, cleaned, and stored at the trial site itself.

Site Security:
[Describe measures in place to ensure security of the trial site to prevent incursion by humans or animals. Measures may include fencing, security patrols, lockable gates, etc…]

The CFT is within the IITA campus in Ibadan that is fenced along the entire perimeter and has restricted access to only authorized personnel.
The CFT site is surrounded by a two-meter high chain-linked fencing buried in the soil, and has a locked gate to prevent unauthorized access by people or animals (see annex A).
A guardhouse is also made available at the gate to allow for 24-hour security (see annex A). To ensure that material confinement standards are met, only approved personnel will have access to the area. A logbook will be maintained to keep a record of all visitors to the site and access will be allowed only to authorized personnel.

6. Records, Personnel, and Planning

Records and Documentation:
[Describe measures in place to ensure adequate documentation of all confinement measures and data requirements as described herein.]

All completed material transfer agreement forms will be incorporated into the compliance binder maintained at the CFT site and will be available at all times for review by Nigerian biosafety inspectors and regulators.
The **compliance forms** to be completed and maintained will include:
Material Transfer forms, to document movement of the plants:
To IITA screenhouse from ETH Zürich, Switzerland.
To the IITA CFT site from the IITA screen house.
Confined Field Trial form, to document the exact number of transgenic cassava plants of each modified line planted in soil in the CFT site, the exact number and purpose of any plants not planted but retained, and the sterilization and/or destruction of any shipping containers.
Weekly Flower Bud Removal form, to document the weekly inspection for flower buds starting from CFT initiation until CFT termination, as well as to document flower bud removal and destruction in the incineration pit. An additional document will be created to summarize the information from the weekly flower bud removal.
Monthly Isolation Monitoring form, to document the monthly inspection of the 100 m isolation distance, as well as to document the destruction of any prohibited plants found to be within the isolation area.
Fertilizer, pesticide and insecticide usage form, to document all fertilizer, pesticide, and insecticide application during and after the CFT
Incident and Corrective Action form, in case of any breach of confinement, IITA IBC and National Biosafety office will be immediately notified by phone of the incident and the corrective action form will be completed.
Isolation distance monitoring form, to document the weekly inspection of the isolation distance and ensure that no cassava plant is established within this area.
Harvest and Destruction form, to document the harvest and destruction of the test plants and the spreader rows.
Post-Harvest Volunteer Monitoring form, to document the monthly post-harvest inspection of the CFT site for living cassava plants and destruction of volunteers.
Transfer to BL2 screen house and laboratory form, to document the harvested material from the field that will be grown in screen house or moved to the laboratory for further analysis at IITA.

The **data forms** to be completed and maintained will include:
Record of Plants in the screen house form, to document and monitor the general health of the plantlets during the hardening period.
CMD infection evaluation form, to document occurrence of CMD infection and severity (1-5 scale scoring) on each of the individual plants within the plots on bi-weekly basis for the entire duration of the experiment.
Monthly Plot Observations form, to record data on plant height, occurrence and severity of cassava bacterial blight, anthracnose, infestation by green mites, whiteflies and mealybugs, as well as any general comments.
Meteorological Data form, to report rainfall, relative humidity and temperature recorded daily by the weather station in IITA.

Personnel:
[Describe measures in place to ensure that trial personnel will have appropriate education, experience and training to adequately perform assigned duties for confinement and technical requirements of the trial.]

Trial personnel have relevant skills in biotechnology and will be appropriately trained in biosafety to cope with the requirements of this study. The principal investigator of this project has long standing experience in biotechnology and compliance with biosafety regulation. A list of authorized personnel for the different activities related to the CFT will be prepared and stored in the compliance binder.

Contingency Plans:
[Describe planned response to the loss of control or accidental release of genetically modified plant material, including notification of authorities and the Permit Holder, recovery and disposal of plant material, and any other measures to be taken to mitigate any potential adverse effects.]

In the highly unlikely event of an accidental release of genetically modified plant material, the IITA IBC and the Nigerian Biosafety officials will be notified immediately and will receive a written notification within 24 hours of becoming aware of the accident. Biosafety inspectors will accompany the material during each stage of transport. An incident and corrective action form will be completed for each case of accidental release. The completed incident and corrective action forms will be incorporated into the compliance binder maintained at the CFT site.
In the unlikely event that transgenic plantlets fall out of its sealed tube packaging material and carrying bag during the transport process, the plantlets will be immediately recovered and returned to its storage tube which will be then marked for subsequent destruction through incineration. Nigerian biosafety officials will be notified immediately of the occurrence. If any plantlet spills from its pot in the closed carton during transport, it will be similarly recovered and then destroyed.
If any plants are accidentally removed from the trial site after planting, the biosafety regulators will be notified immediately of the event and efforts will be undertaken to recover the material under the guidance of the biosafety desk office.
The biosafety inspectors will also be notified immediately of any unintended violation of reproductive isolation. If the breach in the reproductive isolation is due to a cassava plant flowering inside the CFT site, the 100 m isolation distance will ensure that genetic confinement is maintained.
In the unlikely event of civil unrest or natural disaster that affects the integrity of the CFT, the biosafety regulators will be notified and all the entire experimental materials will be destroyed.

7. Declaration

I hereby certify that the information in the application and all attachments is complete and accurate to the best of my knowledge and belief:

Signature of Principal Investigator for Applying Institution:
Dr. Livia Stavolone

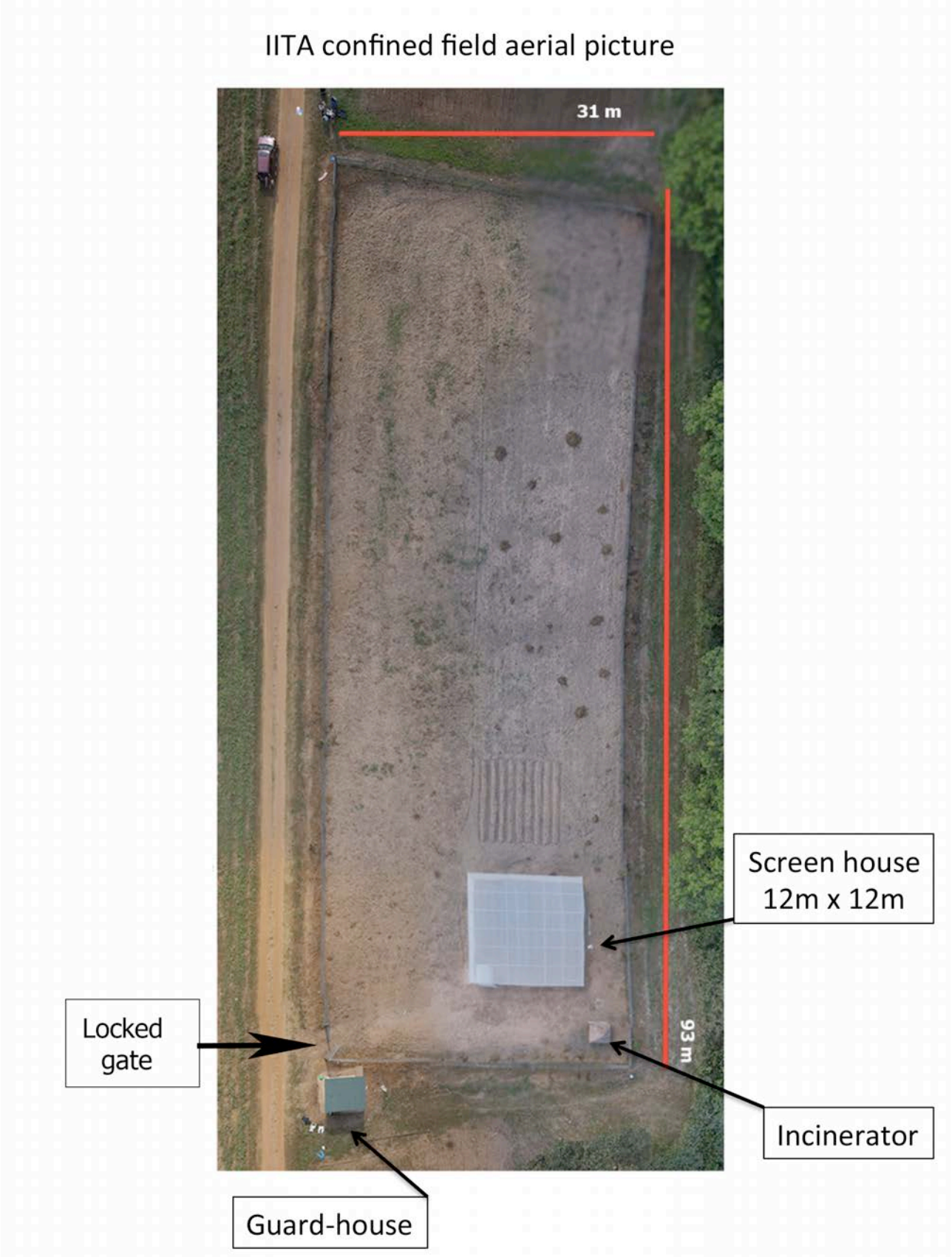
Date:

Signature of Lead Scientist of Collaborating Institution:
Prof. Dr. Samuel C. Zeeman

Date:

ANNEX A

Aerial picture of the site of the confined field trial. Dimensions and critical elements are indicated in the picture.



ANNEX B

AMY3 RNAi lines. Description of vectors and genetic elements used for plant transformation, method of modification, genetic map of the transformation plasmid and illustration of the T-DNA insertion number.

Transformation plasmid: pCAMBIA1301_patatin AMY3 RNAi

(i) Functional cassette:
Promoter : *Solanum tuberosum* class I patatin promoter (position 6-999 bp of the Genbank GQ352473.1)
Gene : *Manihot esculenta AMY3* gene (position 1 to 210 of the Phytozome *Manes.05G097100* coding sequence) in antisens ans sens orientation separated by a plant synthetic intron (57-165 bp of the Genbank M27939 sequence)
Terminator : Nos polyA

(ii) Selectable marker cassette:
Promoter : NOS promoter
Gene : *HPTII*
Terminator : CaMV polyA

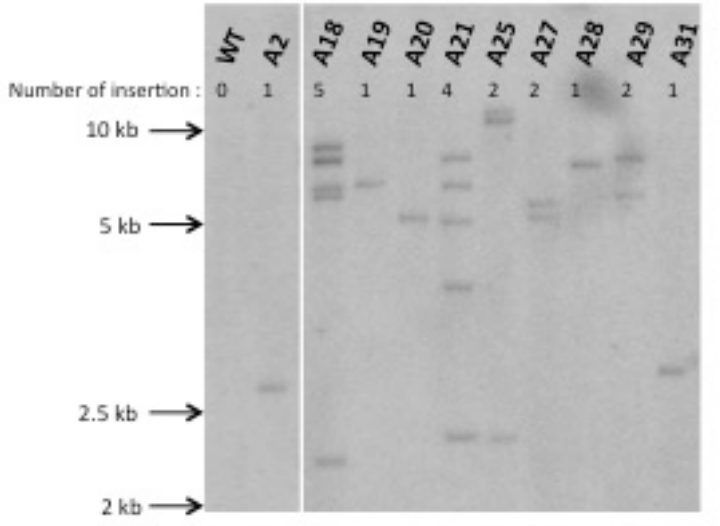
Method of modification

Genetic modification of the cassava cultivar cv60444 has been done following the method described by Bull, *et al*. (1). Briefly, friable embryogenic calli (FEC) have been transformed with A*grobacterium* containing the pCAMBIA1301_patatin AMY3 RNAi binary vector described above. Then hygromycin-resistant embryos have been regenerated and screened to confirm the presence of the full transgene.

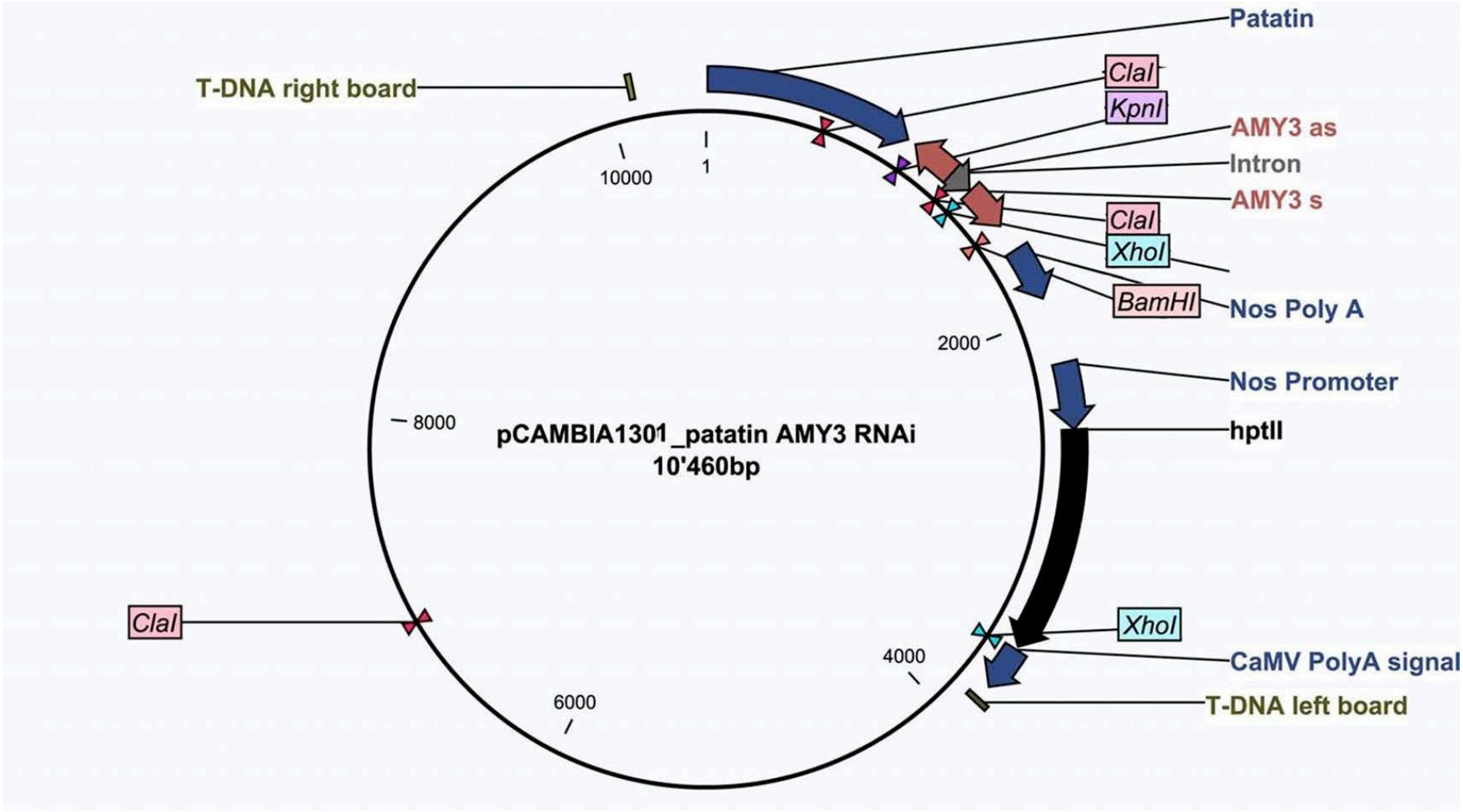
(1) Bull SE, Owiti JA, Niklaus M, Beeching JR, Gruissem W, Vanderschuren H. Agrobacterium-mediated transformation of friable embryogenic calli and regeneration of transgenic cassava. Nat Protoc. 2009;4:1845–1854. doi: 10.1038/nprot.2009.208.

T-DNA insertion number in the AMY3 RNAi lines

Southern blot performed on HindIII digested DNA. ^{32}P-labelled hygromycin probes were used to detect the cassette. Copy number reported in the image below.



Genetic map of the transformation plasmid



ANNEX C

Map of the IITA compound illustrating the habitat around the confined field.
The lake and the forest reserve are clearly well separated from the confined field.