# Implications of epigenetic variation during nodal micropropagation for substantive equivalence analysis: Case study Cassava (*Manihot esculenta* Crantz)

By

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A thesis submitted for the partial fulfilment of the requirements of the Master of Biotechnology
(Plant Biotechnology)

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**Declaration**

I declare that this thesis is a record of original work and contains no material that has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

................................ 18.11.2014 ................................

Shedrack Reuben Kitimu Date

Table of Contents

## Preface

This research was performed over 10 months as part of a Master of Biotechnology (Plant Biotechnology). In accordance with the requirements of the program, the research is presented in the format of a manuscript for submission to a peer-reviewed scientific journal. I have chosen to follow the format of Frontiers in Plant Science. My co-authors for the manuscript are Dr Carlos Rodriguez Lopez, Dr Julian Taylor, Dr Fred Tairo and Prof Mike Wilkinson. Dr Carlos Rodriguez Lopez was the main supervisor who provided all protocols used in this research, ordered some of the reagents that I used in this study and assisted me with the interpretation of the results. Dr Julian Taylor did statistical analysis of peak height and sequencing data. Dr Fred Tairo supervised part of the work done in Tanzania including sample collection and SSR marker assay and Prof Mike Wilkinson provided advice on how the study was designed and also data interpretation.

The manuscript in this thesis is intended as the first draft of a manuscript for future publication, once further data have been collected on genotyping by sequencing (GBS) and methylation sensitive-genotyping by sequencing (msGBS). The word count for the manuscript (excluding references and supplementary material) is 6381. Appendix contains supplementary data.

I have followed instructions to authors for the journal of Frontiers in Plant Science except that I included figure legends in the manuscript easy for examiners to assess and also I have used double space in each paragraph and also between paragraphs in order to satisfy the thesis guidelines for the Master of Biotechnology (Plant Biotechnology) program.

**Manuscript**

# Title: Implications of epigenetic variation during nodal micropropagation for substantive equivalence analysis: Case study Cassava (*Manihot esculenta* Crantz)

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**Key words:** Epigenetics, Transgenic crop, quarantine, Phytosanitary, tissue culture, *in vitro,* micro propagation.

## Abstract

Transgenic plants are rapidly being adopted in parts of the world as a result of increased food demand due to growing population and decrease in agricultural production following dramatic changes in environment. Transgenic crops are accepted for import for food and feeds use and for release into the environment in different countries in the world. For vegetative propagated plants, there is always a phytosanitary concern for international exchange of materials, which require the use of tissue culture as a means to produce virus free plantlets that can then be transported across international boundaries without presenting a quarantine hazard. Although *in vitro* propagation systems that are based on the use of micro cuttings are widely viewed as being analogous to field cuttings, much is not known about the possible epigenetic changes induced by this type of micropropagation. In this study, we survey for epigenetic changes during propagation by node culture and by field cuttings of five cassava (*Manihot esculenta* Crantz) varieties. Genome-wide DNA methylation profiles revealed by Methylation Sensitive Amplified Polymorphism (MSAP) uncovered consistent epigenetic differences between clonal materials recovered from *in vitro* nodes and from those produced from stem cuttings. Also, the observed epigenetic variance between different organs from the same plant was greater between tissues from field grown plants than that observed between organs secured from *in vitro* propagated plants. In this study we uncover differential methylation markers at several loci that could be implicated at organ differentiation and maturation in cassava. We explore the significance of these findings when seeking to predict potential effects to transgenic cassava when imported in tissue culture form and its consequences to the crop physiology and performance during field trials before it is proved substantially equivalent to their traditional local isogenic comparators.

## 1. Introduction

Transgenic plants are rapidly being adopted in parts of the world as a result of increased food demand due to growing population and decrease in agricultural production following dramatic changes in the environment including the spread of viral diseases of important crops (Toenniessen et al., 2003;Chakraborty and Newton, 2011). These factors are especially acute in the sub-Saharan region since it remains the region with biggest imbalance between food availability and population increase in the world (Clover, 2003). Successful adoption of this technology depends on availability of enough information regarding the safe use of the crops resulting from the use of genetic engineering (Obonyo et al., 2011).

Cassava (*Manihot esculenta* Crantz), one of the predominant food crops grown in Tanzania, is attacked by two important viral diseases namely cassava mosaic virus disease (CMVD) and cassava brown streak virus disease (CBSVD). Both diseases greatly affect the crop production (Thresh et al., 1997;Owor et al., 2004;Patil and Fauquet, 2009;Alabi et al., 2011). In sub Saharan Africa, Kenya and Uganda have already tested transgenic CMVD and CBSVD-resistant varieties under greenhouse conditions while Tanzania has initiated arrangements for transforming the crop (Legg et al., 2000). For a successful management of these devastating diseases it would be necessary to disseminate the resistant germplasm of cassava to pandemic areas (Legg et al., 2000), leaving Kenya and Uganda as main sources of transgenic cassava for Tanzania through importation.

Although legislation governing the introduction, testing and release of transgenic plants is still being developed in the region (Adenle, 2011), there is always a phytosanitary concern for international exchange of materials which require the use of tissue culture as a means to produce virus free plantlets that can then be transported across international boundaries without presenting a quarantine hazards (Frison, 1994). From a phytosanitary point of view it is preferred that germplasm is shipped in *in vitro* culture (Kahn, 2012). This means that imported transgenic crops would have been propagated *in vitro* while its local isogenic comparator to which it will be compared during field trials as regulations require would have been propagated through conventional stem cuttings.

In contrast to tissue culture which involve tissue de-differentiation and callus formation which in turn results to a number of genetic and epigenetic changes in regenerants referred to as somaclonal variation (Peraza-Echeverria et al., 2001), micropropagated plants using micro cuttings are widely viewed as being genetically analogous to field cuttings (Kahn, 2012). However, no much is known about the epigenetic variations that can be caused by micropropagating plants in tissue culture by continual growth of differentiated tissues under media.

Epigenetics describes the study of stable alterations in gene expression which occur without any alteration in the underlying DNA sequence and that arise during development and cell proliferation (Meyer et al., 2013). This inheritable information is caused by changes in DNA methylation of cytosines at the 5 position of the pyrimidine ring (5-Me-C) and/or histone modification at their terminal amino acids (Bender, 2004;Ballestar, 2011).

For a transgenic crop to be commercially released, it has to be submitted to regulatory bodies for approval where it has to meet a set of established safety requirements. A main principle and regulatory tool for the safety assessment of transgenic crops is the concept of substantial equivalence (Obonyo et al., 2011;Prochnik et al., 2012). It follows that for the transgenic crop to be considered safe, it has to be substantially equivalent to its traditional isogenic comparator following a comparative approach (Kuiper et al., 2002). In this case, the imported transgenic crops have to be assessed by comparing it to their local isogenic comparators.

Clonal propagation is potential to impact on agronomic performance and nutritional capacities of plants by changing epigenetic control system that may alter gene expression (Vázquez and

Linacero, 2010). This could potentially generate variations which are independent of the variations caused by transgenes introduced into the plants. The aim of the current study therefore was to generate epigenetic profiles using methylation sensitive amplification polymorphism (MSAP), to identify and estimate epigenetic distance and hence epigenetic variations existing between different tissues and genotypes of *Manihot esculenta* Crantz propagated through micro cuttings under *in vitro* culture and stem cuttings under field conditions in order to assess genes regulatory changes associated with propagation history of the plant.

## 2. Materials and Methods

### 2.1.1. Plant materials

Five varieties of cassava (*Manihot esculenta* Crantz) namely Kiroba, Kizimbani, Kibandameno, Mfaransa and Mzungu were collected for this study. All varieties were grown in both *in vitro* and field conditions. *In vitro* samples were obtained from the tissue culture facility of the Mikocheni Agricultural Research Institute (MARI) Dar es Salaam, Tanzania and propagated as described by (Konan et al., 1997). Samples collected in the field were grown at the Sugarcane Research Institute-Kibaha (SRI-KIBAHA), Tanzania. Three replicates from three different tissues (i.e. young leaf, fully expanded leaf and root) were collected from each plant. All samples were placed in small envelopes, transported to the laboratory and stored at -80$^0$C until used for DNA isolation.

### 2.1.2. DNA Isolation

DNA was extracted from all tissue samples following the protocol of Dellaporta et al. (1983) and using a DNeasy plant mini kit (Qiagen 69106) according to the manufacturers' instructions. Two extraction protocols were used to ensure that enough pure DNA is obtained for SSR markers analysis at MARI and MSAP analysis and DNA sequencing (not presented here) at Adelaide University. DNA concentration and quality was estimated using a Nano Drop 1000 Spectrophotometer (Thermo Scientific). DNA was then precipitated and dried for transport to the

Plant Research Centre in Adelaide, Australia. DNA was finally re-suspended in nuclease free water (Sigma), and re-quantified using the Thermo Scientific Nano Drop$^{TM}$ 1000 Spectrophotometer. DNA concentrations were standardized to produce working solutions of 10ng/µl.

## 2.2. Genetic identification of *M. esculenta* Crantz cultivars

Four SSR primer pairs designed for *M. esculenta* Crantz (Mba et al., 2001) and showing high levels of polymorphism according to Moyib et al. (2007) were used to generate genetic fingerprints from two randomly selected samples (one grown in the field and one grown *in vitro*) from each cassava cultivar. Resultant fingerprints were used to confirm the genetic backgrounds and to estimate the genetic similarity between samples. For all primer pairs, the forward primer was labeled with FAM, HEX or NED reporter molecule (Applied Biosystems, Inc, Carlsbad, California, USA) to allow product detection during capillary electrophoresis (For primer information see Table 1). PCR reactions were carried out in a ThemolyneAmplitron 11 Thermocycler as described by Moyib et al. (2007) except that the touch down PCR was not performed. Initial denaturation was carried for 5 minutes, followed by 36 cycles consisting of 45 s at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were separated for visualization on 2% agarose gel stained with Ethidium bromide (0.5 µg/mg) run at 100V for 45 minutes using 1X Trimas Base EDTA (TBE) buffer. After the presence of bands was confirmed, separation was conducted on an ABI PRISM 3130 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols.

**Table 1:** SSR markers used in this study.

| SSR allele | Repeat types | Forward primer sequence | Reverse primer sequence | Reporter Molecule |
|---|---|---|---|---|
| SSR45 | $(CT)_{27}$ | TGAAACTGTTTGCAAATTACGA | TCCAGTTCACATGTAGTTGGCT | FAM |
| SSR50 | $(CA)\ 6(N)\ 6(GA)_{31}$ | CCGCTTAACTCCTTGCTGTC | CAAGTGGATGAGCTACGCAA | HEX |
| SSR51 | $(CT)_{11}CG(CT)_{11}(CA)_{18}$ | AGGTTGGATGCTTGAAGGAA | GGATGCAGGAGTGCTCAACT | NED |
| SSR100 | $(CT)\ 17TT\ (CT)_{7}$ | ATCCTTGCCTGACATTTTGC | TTCGCAGAGTCCAATTGTTG | FAM |

### 2.3. Methylation Sensitive amplification Polymorphism (MSAP) procedure

A modification of the MSAP technique as described by Reyna-Lopez et al. (1997) and adopted by Rodríguez López et al. (2012), was used. The technique involves the digestion of genomic DNA with a isoschizomer pair of enzymes that present different sensitivity to DNA methylation (s), ligation of adaptors and selective PCR amplification with primers complementary to the adaptors but with unique 3' overhangs.

#### 2.3.1. Restriction/Ligation

Genomic DNA was digested with 1U of two methylation-sensitive isoschizomers (*Msp*I or *Hpa*II) as frequent cutters, each in combination with 5U of the rare cutter (*EcoR*I). Generated restriction products were then ligated on the same reaction to 1µl rare (*EcoR*I) (5uM) and 1µl (5uM) frequent (*Msp*I or *Hpa*II) cutter double-stranded DNA adapters (Table 2) using 1 cohesive-end ligation unit (or 0.0 15 Weiss units) T4 DNA ligase with 1.1 µl of T4 DNA ligase buffer in a final volume reaction of 11µl. Both isoschizomers recognize the same sequence (5'-CCGG-3') but differ in their sensitivity to DNA methylation. Comparison of the two profiles for each individual allowed assessment of the methylation state of the restriction sites. Methylated CpG are restricted by *Msp*I only, where hemimethylated CpCpG sites are restricted by *Hpa*II.

Sites that are hypermethylated (i.e., both at the internal and external Cs), and sites that are fully methylated at the external Cs (i.e., on both strands) are not cut by either enzyme, whereas sites that are free from methylation are restricted by both.

### 2.3.2. Preselective amplification

In this step, 1µl of each restriction/ligation reaction was incubated with 0.25µl of PreAmp*Hpa*II/*Msp*I and 0.05µl of PreAmp*EcoR*I (Table 2) primers (all primers at 10 uM) and 6.25µl of 2X BioMix™ (Bioline) in a final volume of 12.5µl supplemented with 0.1 µl of 1mg/ml BSA. PCR conditions were 2 min at $72^{0}$C followed by 30 cycles of $94^{0}$C for 30 s, $56^{0}$C for 30 s and $72^{0}$C for 2 min with a final extension step of 10 min at $60^{0}$C. Representatives of preselective amplification PCR products (3µl) were separated on a 2% w/ v agarose gel by electrophoresis to confirm that PCR amplification was successful.

### 2.3.3. Selective amplification

This amplification aims to reduce the number of fragments generated during the digestion and pre-amplification steps. In this study, a preliminary pilot study was carried to select the best primer combinations. In brief, 3 selective primer combinations comprising one rare primer (*EcoR*I5) and three frequent primers, namely *Hpa*II2.2, *Hpa*II2.3, and *Hpa*II2.4, were combined and tested in a subset of twenty-seven samples comprising nine populations selected to cover most of the crop's distribution in Tanzania. The whole population of samples was then amplified using the best primer combinationion (*Hpa*II2.2// *EcoR*I5). In all cases, selective PCR reactions were performed using 1 µl of preselective PCR reaction product, 0.25µl of frequent primer (6-FAM labelled for detection) and 0.05µl of rare primer (both primers at 10uM), 6.25µl of BioMix™ in a final volume of 12.5 µl supplemented with 0.1 µl of 1mg/ml BSA. Cycling conditions for selective PCR were as follows: 94°C for 2 min, 13 cycles of 94°C for 30 s, 65°C (decreasing by 0.7°C each cycle) for 30 s, and 72°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2min, ending with 72°C for 10 min. As in preselective amplification, selective PCR products (3µl) were separated on a 2% w/ v agarose gel by electrophoresis to confirm correct amplification.

**Table 2:** MSAP primer sequences used for MSAP

| **Oligo name** | **Function** | **Sequence** |
|---|---|---|
| Ad *Hpa* II/*Msp* I | Reverse Adaptor | GACGATGAGTCTAGAA |
| Ad. *Hpa*II/*Msp*I | ForwardAdaptor | CGTTCTAGACTCATC |
| Ad. *EcoR*I | Reverse Adaptor | AATTGGTACGCAGTCTAC |
| Ad *EcoR* I | ForwardAdaptor | CTCGTAGACTGCGTACC |
| Pre. *EcoR* I | Preselective primer | GACTGCGTACCAATTCA |
| Pre. *Hpa*II/*Msp*I | Preselective primer | GATGAGTCCTGAGCGGC |
| *EcoR*I5 | Selective primer | GACTGCGTACCAATTCACA |
| *Hpa*II 2.2 | Selective primer | GATGAGTCCTGAGCGGCC |
| *Hpa*II 2.3 | Selective primer | GATGAGTCCTGAGCGGCG |
| *Hpa*II 2.4 | Selective primer | GATGAGTCCTGAGCGGCT |

### 2.4. Sample fractioning

Capillary separation was performed at the Australian Genome Research Facility Ltd, Adelaide South Australia.

### 2.5. Statistical analysis

#### 2.5.1. Genetic identification of *M. esculenta* Crantz cultivars

The MultiVariate Statistical Package (MVSP) was used to analyze all scored alleles resulted from SSR markers used here in order to analyze the genetic similarity between samples grown *in vitro* and in the field using cluster analysis under Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method.

#### 2.5.2. Analysis of epigenetic variability

MSAP profiles were visualized using GeneMapperSoftware v4 (Applied Biosystems, Foster City, CA). Two matrices were generated containing allelic information 1) a presence/absence binary matrix. In this case MSAP fragments with allelic sizes of 100 bp or larger were considered to reduce the potential impact of size homoplasy (Caballero, Quesada, &Rolán-Alvarez, 2008), 2) a matrix containing the peak heights of all fragments with allelic size of 50bp or larger for quantitative analysis.

Different levels of hierarchy were generated to group the samples. First samples were grouped according to the cassava variety they were taken from. Then, samples were separated into field grown and *in vitro* grown samples. Finally, samples were separated into three different tissues (young leaf, mature leave and root).

First, for the analysis of the qualitative data, GenAlex v6.4 software (Peakall and Smouse, 2006) was used to infer epigenetic distances between different cassava tissues within the same cultivar and between cultivars. Pairwise PhiPT (estimation of genetic/epigenetic distance between populations) was calculated using GenAlex v. 6.4 software. Then Analysis of Molecular Variance (AMOVA) was performed using the same software to analyse the proportion of variance distributed amongst and within samples and to test the significance of PhiPT between tissues (Michalakis and Excoffier, 1996). An allele frequency table was generated using GenAlex 6.4 to find tissue specific qualitative markers for each cultivar and for all cultivars. Finally, the visualization of the patterns of tissue epigenetic variations in this study was done by constructing a Principal Coordinates Analysis (PCoA).

Second, for quantitative analysis, peak height data was initially normalized using the methods derived in Robinson and Oshlack (2010). An analysis of peak heights was then conducted using the methods derived in (Robinson and Smyth, 2007;2008) and computationally implemented in the package edgeR (Robinson et al., 2010) available in the Team (2014).

## 3. Results

### 3.1. Genetic identification of *M. esculenta* Crantz cultivars

After positive PCR amplification was confirmed by visualization on agarose, samples presenting clear amplification were capillary separated and alleles obtained were analyzed using

MultiVariate Statististical Package (MVSP). The number of SSR alleles detected for each primer combination among the 5 cassava varieties ranged from 1 to 2 alleles (Supplementary Table 8). The calculated percentage similarity from similarity matrix generated through cluster analysis using SSR markers ranged from 95.7%to 100%. Pair wise percentage similarities for each pair of variety were: Kibandameno (99.13%), Kiroba (100%), Kizimbani (98.56%), Mfaransa (100%) and Mzungu (97.7%). However, the dendrogram obtained using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis method in the MVSP software package revealed 3 distinct DNA cluster groups at 98.1-percentage similarity (Figure 1). Cluster analysis showed that samples from field and *in vitro* of three out of the five varieties were grouped together. Two of these cultivars (Kiroba and Mfaransa) did not show any detectable *in vitro* culture induced genetic mutation. Conversely, genetic variation was detected between field and their *in vitro* counterparts in two varieties Mzungu and Kibandameno (Figure 2). While Kibandameno field sample showed to be genetically very similar to its *in vitro* counterpart, Mzungu field and *in vitro* samples were significantly different from each other.

**Figure 1**. Genetic similarity between field and *in vitro* samples. Dendrogram showing the calculated genetic similarity obtained from 4 SSR markers in 10 cassava samples from 5 different cultivars grown under field and *in vitro* conditions. Dendrogram is based on UPGMA and generated using MVSP software.

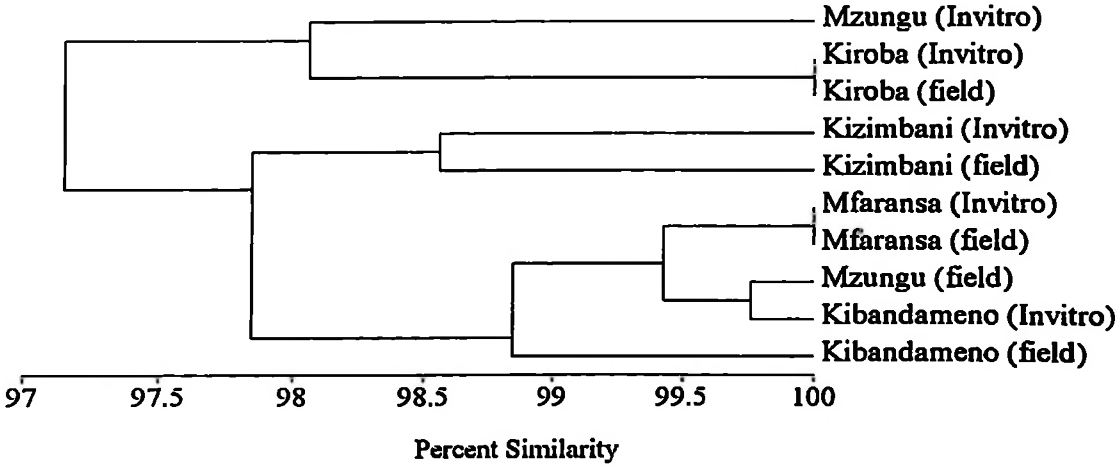
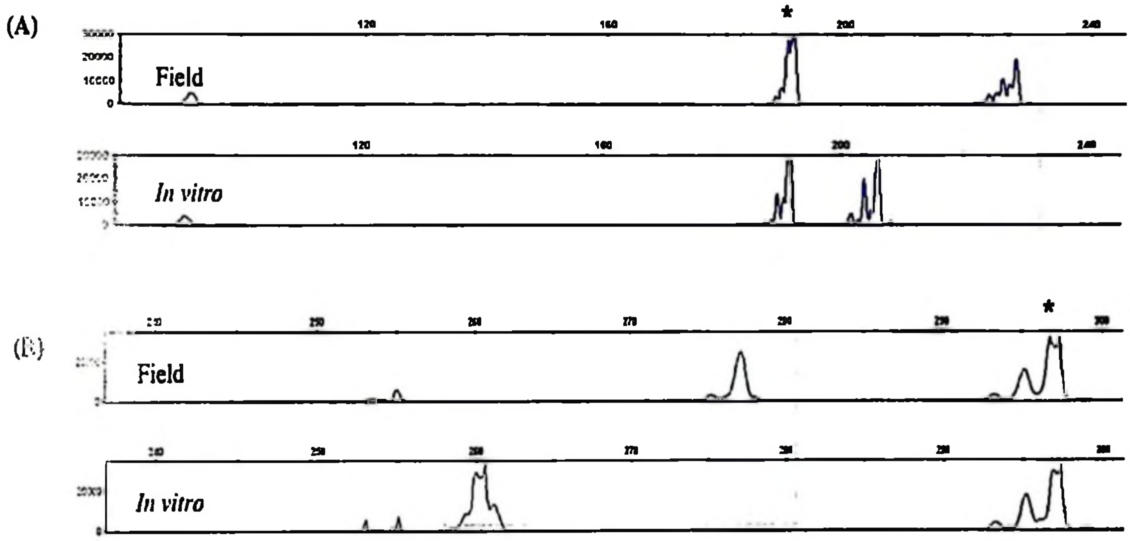


**Figure 2:** Detected polymorphic SSR alleles in Mzungu cultivar. SSR electropherograms showing genetic variation between field cutting and *in vitro* cultured samples of Mzungu cultivar. Horizontal axis represents the estimated fragment size in base pairs and vertical axis indicate the peak height in relative fluorescence units. The variations showed on *in vitro* culture sample results from genetic mutations due to tissue culture conditions. Blue electropherograms (A) were obtained using primer SSR50 while the green electropherograms (B) were generated using primer SSR51. Blue boxes show detected polymorphic alleles while asterisks indicate conserved alleles between field and *in vitro* samples.



### 3.2. Analysis of the epigenetic variability in cassava

MSAP analysis generated 155 epiloci for the 86 samples of ten cassava cultivars used in this study. Further analysis identified differential epimarkers/epiloci from the presence and absence (Table 3) and peak height data (Table 7) that separated *in vitro* from field samples and also different three tissues, root, mature leaf and young leaf of cultivars grown under the two conditions.

**Table 3:** Total qualitative epigenetic epigenetic markers across all cultivars grown under *in vitro* and field conditions. Tissues derived from the field show more epigenetic markers that those derived from *in vitro* condition.

| Cultivar | Field | *In vitro* |
|---|---|---|
| Mzungu | 52 | 24 |
| Kiroba | 47 | 15 |
| Kibandameno | 50 | 14 |
| Kizimbani | 52 | 20 |
| Mfaransa | 32 | 20 |
| Total | 233 | 93 |

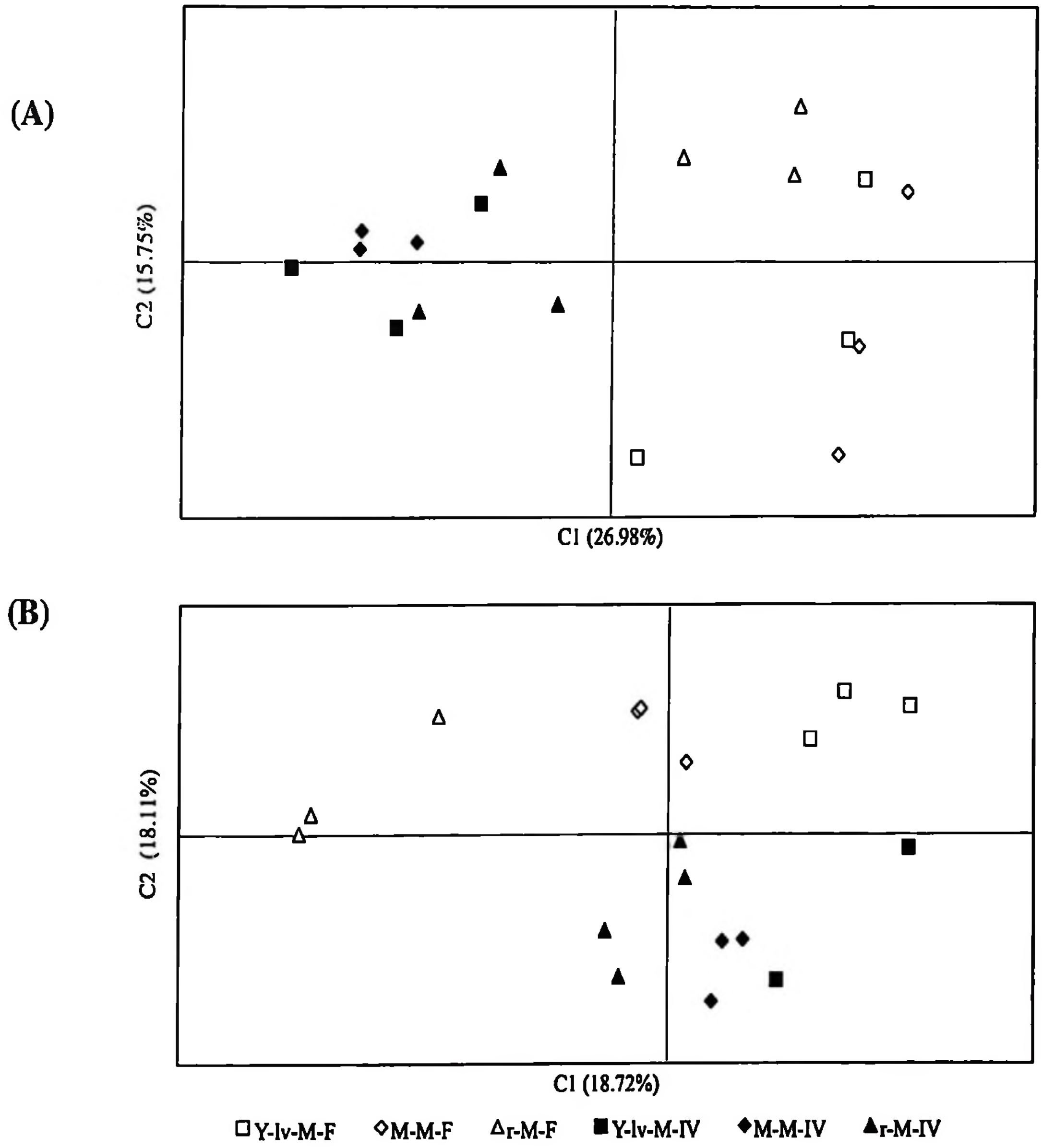
### 3.2.1. Qualitative analysis of epigenetic variability in cassava

PCoA analysis of the presence/absence MSAP profiles generated from *Hpa*II and *Msp*I restricted samples showed clear separation between *in vitro* propagated cultivars and their field counterparts (Figure 3). Observed distances were significant for all cultivars (Table 4). In general, calculated distances between *in vitro* and field samples where higher when samples when restricted using *Msp*I (Table 4).

**Table 4:** Summary of calculated epigenetic distance (*PhiPT*) between *in vitro* and field grown samples. PhiPT values were calculated from MSAP profiles obtained by restricting with *Hpa*II or *Msp*I 9 DNA samples from ten cultivars of five cassava varieties grown *in vitro* or in the field. The *p-values* were calculated based on 999 permutations.

| | *Hpa*II | | *Msp*I | |
|---|---|---|---|---|
| | *PhiPT* | *p-value* | *PhiPT* | *p-value* |
| Mzungu | 0.159 | 0.006 | 0.188 | 0.001 |
| Kiroba | 0.130 | 0.002 | 0.310 | 0.001 |
| Kibandameno | 0.273 | 0.001 | 0.374 | 0.001 |
| Mfaransa | 0.577 | 0.001 | 0.541 | 0.002 |
| Kizimbani | 0.160 | 0.003 | 0.243 | 0.001 |

Fig. 3: Principal component analysis of epigenetic variability in cassava (Kiroba (A) and Mzungu (B)) samples. Principal Coordinate Analysis based on the Euclidean analysis of methylation-sensitive amplified polymorphism (MSAP) obtained from samples from three different tissues of Mzungu and Kiroba cultivar (*in vitro* and field) restricted with *Msp*I and amplified using primer combination H2E5/*Msp*I. Y-lv=young leaf, M= mature leaf, r= root, F=Field and IV=*In vitro*.



In order to investigate which tissue from field plants was epigenetically closer to the *in vitro* culture samples, samples derived from the three tissues grown in the field were compared to all nine samples grown *in vitro* considered as a single group. Whilst all calculated epigenetic distances were significant for all pairs, young leaves and mature leaves tissues from field cuttings were consistently epigenetically closer to bulked *in-vitro* tissues using *Msp*I (Table 5). The same analysis for *Hpa*II where most of the epigenetic distances were not significant has been presented in supplementary materials (Table 9).

**Table 5:** Epigenetic distance between field grown and *in vitro* samples. Calculated pairwise tissue PhiPT values (epigenetic distances) obtained from MSAP profiles obtained by restricting genomic DNA from ten cultivars of five cassava varieties with *Msp*I using GenAlex 6.5.1 AMOVA. Values show the distance between individual tissues of field cutting lines from bulks of *in vitro* propagated lines. YIvF-Iv=distance between field derived young leaves and bulked *in vitro* tissues, MIvF-Iv= distance between field derived mature leaves and bulked *in vitro* tissues and RIvF-Iv distance between field derived root and bulked *in vitro* tissues. Probability values (p) based on 999 permutations are shown beside each distance. Asterisks indicate lower PhiPT values.

| | Kizimbani | | Mzungu | | Kiroba | | Mfaransa | | Kibandameno | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *PhiPT* | p | *PhiPT* | p | *PhiPT* | p | *PhiPT* | p | *PhiPT* | p |
| YIvF-Iv | 0.486 | 0.007 | 0.298 | 0.006 | 0.308* | 0.006 | 0.559* | 0.026 | 0.502* | 0.007 |
| MIvF-Iv | 0.350* | 0.001 | 0.286* | 0.003 | 0.423 | 0.007 | - | - | 0.521 | 0.004 |
| RIvF-Iv | 0.528 | 0.005 | 0.398 | 0.006 | 0.389 | 0.007 | 0.655 | 0.006 | 0.576 | 0.007 |

We observed diffential epigenetic markers, which separate different tissues across all cultivars. There are more tissue differential epigenetic markers between tissues for the cultivars grown on the field than those grown under *in vitro* conditions. General results show that there are as many as twice the differential markers observed in crops grown on field compared to those grown under *in vitro* conditions. This is evident with the PCoA analysis where tissues from *in vitro* propagated cultivars were closer to each other than those of tissues obtained from field cutting propagated ones (Figure 3). Also for each cultivar, we calculated epigenetic distance existing between tissues using samples that were digested using *Msp*I and *Hpa*II enzymes. General findings show that the average epigenetic distance between field cutting derived tissues was higher than that between *in vitro* cultivar derived tissues (Table 6).

**Table 6:** Effect of *in vitro* culture on epigenetic differentiation between tissues. Calculated pairwise PhiPT values (epigenetic distances) between tissues from field and *in vitro* samples obtained from MSAP profiles obtained by restricting genomic DNA from ten cultivars of cassava with *Msp*I and *Hpa*II using GenAlex 6.5.1 AMOVA. Data for Mfaransa mature leaves is missing. Ylv=Young leaves, Mlv=Mature leaves and Rt=Roots.

| **Tissues** | | ***Hpa*II** | | ***Msp*I** | |
|---|---|---|---|---|---|
| | *Cultivar* | *Field* | *Invitro* | *Field* | *Invitro* |
| Ylv-Mlv | ***Kibandameno*** | 0.208 | 0.070 | 0.237 | 0.000 |
| | ***Mzungu*** | 0.000 | 0.042 | 0.239 | 0.192 |
| | ***Mfaransa*** | - | 0.112 | - | 0.209 |
| | ***Kizimbani*** | 0.171 | 0.000 | 0.114 | 0.071 |
| | ***Kiroba*** | 0.259 | 0.178 | 0.260 | 0.118 |
| | **Average** | **0.1595** | **0.0804** | **0.2125** | **0.118** |
| Ylv-*Rt* | ***Kibandameno*** | 0.447 | 0.296 | 0.308 | 0.197 |
| | ***Mzungu*** | 0.000 | 0.162 | 0.461 | 0.178 |
| | ***Mfaransa*** | 0.401 | 0.260 | 0.538 | 0.148 |
| | ***Kizimbani*** | 0.142 | 0.272 | 0.654 | 0.286 |
| | ***Kiroba*** | 0.264 | 0.098 | 0.250 | 0.090 |
| | **Average** | **0.2508** | **0.2176** | **0.4422** | **0.1798** |
| Mlv-Rt | ***Kibandameno*** | 0.196 | 0.301 | 0.049 | 0.362 |
| | ***Mzungu*** | 0.108 | 0.344 | 0.459 | 0.386 |
| | ***Mfaransa*** | - | 0.408 | - | 0.375 |
| | ***Kizimbani*** | 0.263 | 0.139 | 0.550 | 0.250 |
| | ***Kiroba*** | 0.357 | 0.201 | 0.344 | 0.212 |
| | **Average** | **0.231** | **0.2786** | **0.3505** | **0.317** |

### 3.2.2. Quantitative Analysis of epigenetic variability in cassava

Quantitative analysis using edgeR software of differential expression analysis for the peak height data revealed differential epimarkers/epiloci which significantly separate different tissues and cultivars grown under field and *in vitro* conditions (Table 7). All the markers and there corresponding p-values have been included in supplementary materials (Table 10-18). General observation shows that there are more differential epigenetic markers for cultivars propagated by the field cuttings than those propagated under *in vitro* conditions when the genomic DNA was cut using both *Hpa*II and *Msp*I enzymes (Table 7).

**Table 7:** Number of quantitative significantly different epimarkers across all cultivars. Quantitative markers were generated from MSAP profiles peak heights obtained by restricting genomic DNA from five cultivars of cassava (*in vitro* and in the field) with *Msp*I and *Hpa*II. Column Field vs *in vitro* shows the number of significantly different epimarkers between samples of the same cultivar grown *in vitro* or in the field. Columns Tissues (*in vitro*) and Tissues (Field) show the number of significantly different epimarkers tissues grown either in the field or *in vitro*. Significance was calculated using edgeR (P>0.001).

| | *Msp*I | | | *Hpa*II | | |
|---|---|---|---|---|---|---|
| Variety | Field vs *in vitro* | Tissues (*in vitro*) | Tissues (Field) | Fied vs *in vitro* | Tissues (*in vitro*) | Tissues (*Field*) |
| Mfaransa | 6 | 7 | 7 | 0 | 5 | 8 |
| Mzungu | 0 | 9 | 13 | 1 | 5 | 5 |
| Kizimbani | 3 | 1 | 20 | 2 | 5 | 4 |
| Kiroba | 8 | 8 | 7 | 3 | 8 | 6 |
| Kibandameno | 6 | 9 | 1 | 7 | 4 | 11 |
| Total | 23 | 34 | 48 | 13 | 27 | 34 |

So importantly further analysis of peak height revealed two epiloci 55 and 101, which separate all *in vitro* tissues from field tissues in three varieties, Kiroba, Kibandameno and Kizimbani using *Hpa*II and *Msp*I enzymes. While in all cases epilocus 55 was significant when both enzymes were used, epilocus 101 was only significant when *Hpa*II was used while insignificant when *Msp*I was used (Figure 4). This indicates that variations at 55 epilocus is due to both genetic and epigenetic changes while variations due to epilocus 101 was mainly due to epigenetic changes caused by tissue culture conditions.

On the other hand, we identified markers which significantly differentiate tissues in both field and *in vitro* conditions (Figure 5). The rest of results have been presented as supplementary materials (Table 10-18). Some of the markers were only present in tissue culture conditions while other are only identified under field conditions indicating that there are diagnostic markers for the two conditions.

**Figure 4:** *Msp*I (A) *Hpa*II (B) data obtained from peak height data showing two important markers (55 and 101) which separated all *in vitro* tissues from field tissues for the three varieties. Asterisk indicates that there is a significance difference, the black circles are the medians of the sample. The blue circles are outliers that are outside the 1.5 x inter quartile range (IQR) which is shown by the blue box.

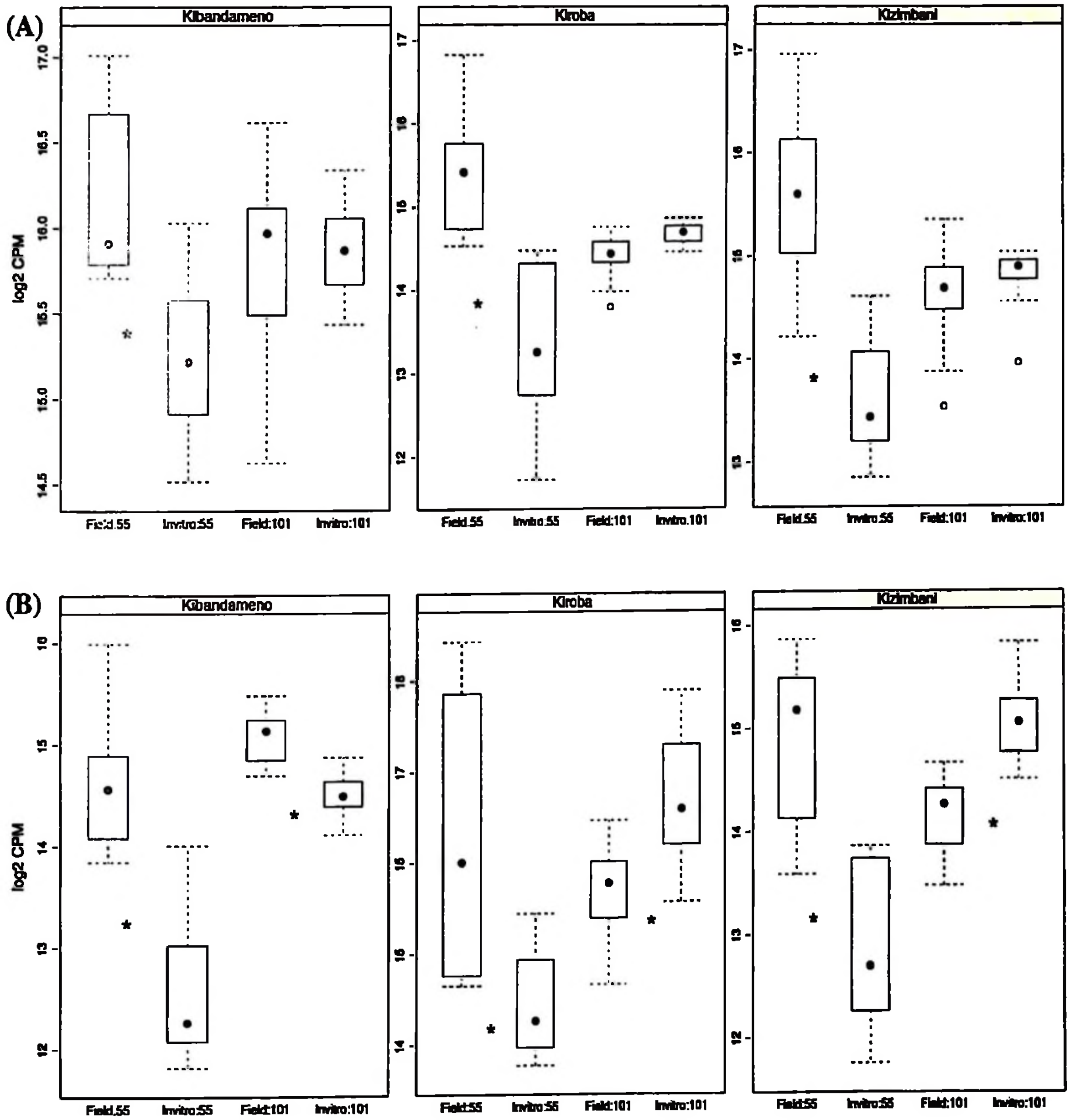
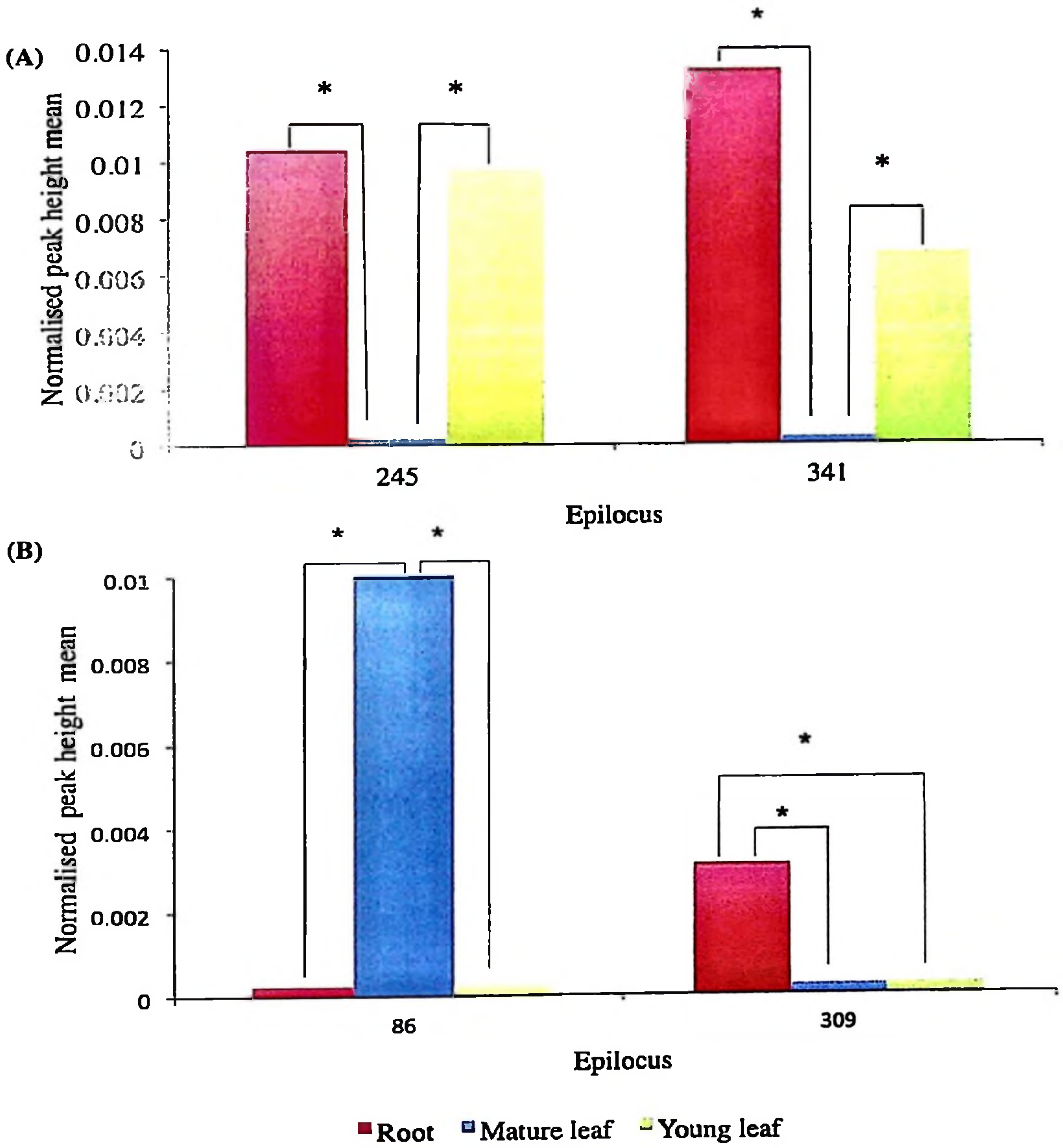


**Figure 5:** Quantitative data showing tissue separations in field (A) and *in vitro* (B) for Kiroba. The black lines proceeded by asterisks show significance difference. The horizontal line show significant epiloci.



## 4. Discussion

Clonal propagation through *in vitro* conditions needs to produce individuals identical to the plant from which they were sub-cultured (Debergh and Read, 1991) although it has been reported that protracted cultures induces the appearance of genetic and epigenetic changes (Dann and Wilson, 2011). Such changes have the potential to lead to phenotypic changes in the propagated clones and eventually alterations in agronomic performance of crops when grown in field conditions (Vázquez and Linacero, 2010). In the current study, we surveyed the possible somaclonal variation induced by micropropagation in cassava. To achieve this we first screened for epigenetic variability between different organs and development stages within plants (i.e, young leaf, mature leaf and root) grown *in vitro* and in the field. We then analyzed the genetic and epigenetic variations existing between *in vitro* propagated cassava lines and their genetic counterparts grown in the field.

Genetic similarity between field cuttings and their *in vitro* counterparts ranged from 97.7% to 100%. Cultivars Kiroba and Mfaransa showed 100% similarity while Mzungu showed the lowest level of similarity (97.7%). This observed genetic variation could have three potential explanations 1) genetic mutations caused by tissue culture; 2) since *in vitro* culture samples were not regenerated directly from their field counterparts, the observed genetic variability could be explained by the normal genetic diversity of the crop or 3) the pairwise compared samples could belong to different genotypes and therefore were originally mislabeled. With estimates of up to 40%, mislabeling of plant accessions is considered one of the main problems affecting germplasm collections around the world (Irish et al., 2010).This could potentially create a serious threat for biotechnological and breeding programs that require the use of elite cassava genotypes (Munoz et al., 2014). These findings should call the attention of experts involved in Tanzania's cassava research programs to further investigate this.

Although mislabeling of plants during vegetative or *in vitro* propagation cannot be ruled out

(Seefelder et al., 2000) in this study, the genetic variations here observed may be due to mutations induced by tissue culture conditions. Tissue culture conditions have the potential to induce genetic mutations (Phillips et al., 1994;Kaeppler et al., 2000;Jin et al., 2008) following regeneration of plants. The regeneration systems with a higher reported level of genetic mutation are the ones that include a dedifferentiation stage through callus formation (Arene et al., 1993;Bregitzer et al., 2002). *In vitro* plants materials used in this study were obtained from nodal culture as in Konan et al. (1997). Micropropagated plants using this approach have been reported to present high levels of genetic stability (Lata et al., 2010). However, the extent of genomic instability does not depend only on the explant type or *in vitro* system used but also on other factors such as genotype, genome size, age of the culture and nature and/or concentration of the exogenous growth factors used in nutrient media (Bairu et al., 2011). An *in vitro* culture origin for the genetic variations observed here cannot be discarded and may therefore be genotype dependent, culture age or nutrient media concentration.

Using MSAP we identified several qualitative and quantitative differential epigenetic markers that separate both organs and developmental stages from the same plant and *in vitro* propagated samples from field samples. Our results show a higher level of epigenetic differentiation between tissues of plants grown in the field both using a qualitative (Table 3) and a quantitative approach (Table 7) than for those grown under *in vitro* conditions. This suggests that tissues sampled from the field have undergone a much higher epigenetic differentiation than their *in vitro* propagated counterparts. Furthermore, calculated epigenetic distances (PhiPT) showed that *in vitro* samples are always epigenetically closer to either young or mature leaves of their field counterparts. The cassava micropropagation protocol followed in this study uses lateral meristems as source material (Konan et al., 1997). Such meristems contain primordial leaves but no roots. Therefore, tissues generating new leaves do not need to undergo a dedifferentiation stage while those generating roots do. This supports previous studies that show that epigenetic profiles of *in vitro* propagated tissues tend to retain some features of the source tissue (Rodriguez Lopez et al., 2010).

Finally, comparison between *in vitro* and field samples revealed a significant epigenetic distance ($p \leq 0.007$) between samples grown under both conditions. These findings correlate with previous studies that show that long-term nodal tissue culture induces epigenetic variations in potato somatic hybrid regenerants (Tiwari et al., 2007). More specifically, the analysis of peak height revealed two epiloci (55 and 101), which separate all *in vitro* tissues from field tissues in three varieties, Kiroba, Kibandameno and Kizimbani. While in all cases epilocus 55 was significantly different between *in vitro* and field lines when both enzymes were used, epilocus 101 was only significant when *Hpa*II was used. MSAP fragment analysis can differentiate between genetic and epigenetic polymorphisms. In brief, a polymorphic marker for both enzymes (*Hpa*II and *Msp*I) when comparing two samples could be due to a genetic or an epigenetic change. Conversely, a polymorphic marker detected by only one of the enzymes can only be epigenetic in nature (Pérez-Figueroa, 2013). This indicates that variations at 55 epilocus could be explained by both genetic and epigenetic changes while variations due to epilocus 101 was due to epigenetic changes caused by tissue culture conditions. Furthermore, since the chance of a mutation happening exactly at the same location in more than one occasion is extremely low (Rodriguez Lopez et al., 2010) combined with the fact that these two polymorphic markers were found in three different cultivars indicates that these are epigenetic mutations induced by *in vitro* culture. These markers could potentially be molecular targets to study the epigenetic bases of the mechanisms regulating plant cell differentiation both *in vitro* and during normal plant development.

There is evidence that gene expression can be tissue specific depending on their role in the course of plant development (Grimplet et al., 2007). DNA methylation has a very important role on gene expression, plant development and tissue differentiation (Messeguer et al., 1991). It is therefore, tempting to speculate that *in vitro* propagated plants that partially maintain the epigenetic profiles of their source tissues might have their agronomical properties compromised when grown in the field. If these markers are maintained when the *in vitro* propagated lines of cassava are later grown in field conditions, it is our concern that there may be notable effects in phenotypic and hence agronomic performance. This will potentially affect the transgenic plants in terms of agronomic performance and changing nutritional composition when compared to their conventional isogenic comparators, which have always being propagated as stem cuttings in the field. This could potentially pose confounding effects to transgenic crop that will be imported and kept under field trials before it is environmentally released. Scientists suggest that the presence of transgene in the plant is the main cause of crops variations during field evaluation and agronomic performance (Oard et al., 1996;Barro et al., 2002;Song et al., 2011;Pons et al., 2012) but here we also suggest that tissue culture propagation could also be the cause of phenotypic variations that may eventually disqualify any micro propagated imported transgenic crop.

Results obtained in this study raise the concern on whether the resulting regenerants will be substantially equivalent to their isogenic counterparts independently of a transgene. To our knowledge this is the first study to show previously ignored evidence that the lack of substantial equivalence of genetically modified organisms might be due partially to the propagation system used. This applies to transgenic cassava that may be imported to Tanzania from either Kenya or Uganda which will require the use of micropropagation system as it is required by phytosanitary regulations. It is clear that more study is required for extensive sequencing of the MSAP fragments and differential gene expression analyses in order to examine genomic regions most affected by epigenetic changes and eventually identifying genes affected. More importantly growing the *in vitro* plants in field conditions and see whether the observed *in vitro* culture induced epigenetic markers can be recovered.

## 5. Manuscript Publication

This manuscript has been pre-accepted for publication in the journal Frontiers in Plant Science.

Research Topic Title: Recent Advances of Epigenetics in Crop Biotechnology.

Topic Editor(s): Clelia De-la-Peña, Raúl Alvarez-Venegas and Christopher Cullis

Article Submission Deadline: 01 Apr 2015.

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**Appendix: Supplementary materials.**

**Table 8:** The SSR alleles (in base pair) detected for all primer combinations used for ten cassava cultivars of five varieties. For each primer pair, one or two alleles were scored.

| | SSRY100 | | SSRY50 | | SSRY51 | | SSRY45 | |
|---|---|---|---|---|---|---|---|---|
| Kibandameno (field) | 191 | 245 | 255 | 255 | 277 | 297 | 191 | 191 |
| Kibandameno (*Invitro)* | 201 | 222 | 255 | 255 | 277 | 297 | 191 | 191 |
| Kiroba (field) | 191 | 191 | 248 | 251 | 256 | 277 | 191 | 191 |
| Kiroba (*Invitro)* | 191 | 191 | 248 | 251 | 256 | 277 | 191 | 191 |
| Kizimbani (field) | 191 | 222 | 255 | 255 | 277 | 260 | 191 | 206 |
| Kizimbani (*Invitro)* | 201 | 222 | 227 | 255 | 277 | 260 | 191 | 191 |
| Mfaransa (field) | 201 | 222 | 255 | 255 | 260 | 297 | 191 | 191 |
| Mfaransa (*Invitro)* | 201 | 222 | 255 | 255 | 260 | 297 | 191 | 191 |
| Mzungu (field) | 201 | 213 | 255 | 255 | 277 | 297 | 191 | 191 |
| Mzungu (*Invitro)* | 191 | 191 | 255 | 255 | 260 | 297 | 191 | 227 |

**Table 9:** Epigenetic distance between field grown tissues and *in vitro* samples

| | Kizimbani | | Mzungu | | Kiroba | | Mfaransa | | Kibandameno | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *PhiPT* | *p-values* | *PhiPT* | *p-values* | *PhiPT* | *p-values* | *PhiPT* | *p-values* | *PhiPT* | *p-values* |
| YlvF-Iv | 0.222 | 0.119 | 0.235 | 0.048 | 0.150 | 0.030 | 0.621 | 0.010 | 0.391 | 0.006 |
| MF-Iv | 0.327 | 0.009 | 0.370 | 0.018 | 0.247 | 0.008 | | | 0.296 | 0.005 |
| RF-Iv | 0.109 | 0.076 | 0.207 | 0.013 | 0.062 | 0.147 | 0.597 | 0.007 | 0.372 | 0.003 |

Calculated pairwise tissue PhiPT values (epigenetic distances) obtained from MSAP profiles obtained by restricting genomic DNA from five cultivars of cassava with *Hpa*II using GenAlex 6.5.1 AMOVA. Values show the distance between individual tissues of field cutting lines from bulks of *in vitro* propagated lines. YlvF-Iv=distance between field derived young leaves and bulked *in vitro* tissues, MF-Iv= distance between field derived mature leaves and bulked *in vitro* tissues and RF-Iv distance between field derived root and bulked invitro tissues. Probability values (p) based on 999 permutations are shown beside each distance.

**Table 10:** Quantitative epimarkers swhich separate different tissues for Kibandameno cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kibandameno(Field): Mature Leaf vs Root | 63.18 | 9.972429659 | 0.001187315 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 63.18 | 9.839917242 | 0.000371709 |
| Kibandameno(Field): Mature Leaf vs Root | 69.117253 | 12.75448311 | 8.50314E-05 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 69.117253 | 13.33436733 | 0.000214146 |
| Kibandameno(Field): Mature Leaf vs Root | 76.843673 | 12.33488548 | 0.001059538 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 76.843673 | 12.97554812 | 0.004608538 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 86.680869 | 12.77340268 | 0.000419844 |
| Kibandameno(Field): Root vs Young Leaf | 86.680869 | 14.58287658 | 2.14016E-18 |
| Kibandameno(Field): Root vs Young Leaf | 172.4963958 | 8.563930013 | 0.002407581 |
| Kibandameno(Field): Mature Leaf vs Root | 263.978125 | 10.30067715 | 2.67489E-06 |
| Kibandameno(Field): Root vs Young Leaf | 263.978125 | 10.23849032 | 1.53044E-07 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 351.8970308 | 14.12311211 | 8.39852E-05 |
| Kibandameno(Field): Root vs Young Leaf | 351.8970308 | 14.33483726 | 2.78008E-07 |
| Kibandameno(Field): Root vs Young Leaf | 435.0555128 | 8.345454495 | 0.005720588 |
| Kibandameno(Field): Root vs Young Leaf | 457.7534591 | 8.197199549 | 0.008201916 |
| Kibandameno(Field): Root vs Young Leaf | 458.9518627 | 8.068595848 | 0.011659063 |
| Kibandameno(Field): Root vs Young Leaf | 508.149547 | 8.067287583 | 0.011463058 |
| Kibandameno(Invitro): Mature Leaf vs Root | 86.680869 | 13.98291025 | 0.00093436 |
| Kibandameno(Invitro): Root vs Young Leaf | 86.680869 | 13.94616983 | 0.001374832 |
| Kibandameno(Invitro): Root vs Young Leaf | 288.6200676 | 8.114924413 | 0.012863968 |
| Kibandameno(Invitro): Root vs Young Leaf | 290.6145989 | 8.13019579 | 0.011237596 |
| Kibandameno(Invitro): Mature Leaf vs Root | 309.8511607 | 9.618987126 | 6.94776E-05 |

**Table 11:** Quantitative epimarkers swhich separate different tissues for Kiroba cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kiroba(Field): Mature Leaf vs Root | 154 | 9.4738814 | 0.005436594 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 154.5674187 | 10.96222961 | 0.002714439 |
| Kiroba(Field): Mature Leaf vs Root | 157.934391 | 9.555751315 | 0.003358202 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 157.934391 | 10.76813108 | 0.002139413 |
| Kiroba(Field): Mature Leaf vs Root | 161.1483275 | 10.13039125 | 0.000527766 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 161.1483275 | 10.06605837 | 0.013037778 |
| Kiroba(Field): Mature Leaf vs Root | 245.5640164 | 10.57347977 | 0.001493162 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 245.5640164 | 10.71303489 | 0.003200879 |
| Kiroba(Field): Mature Leaf vs Root | 341.4326428 | 10.98218714 | 0.001789805 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 341.4326428 | 10.17383884 | 0.001679632 |
| Kiroba(Field): Mature Leaf vs Root | 378.5835476 | 9.191022155 | 0.008458824 |
| Kiroba(Invitro): Mature Leaf vs Root | 55.240281 | 12.65480416 | 0.012053232 |
| Kiroba(Invitro): Mature Leaf vs Root | 69.117253 | 10.48764075 | 1.51278E-12 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 69.117253 | 10.14219076 | 6.3765E-07 |
| Kiroba(Invitro): Mature Leaf vs Root | 86.680869 | 16.28063569 | 3.19406E-07 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 86.680869 | 15.84575458 | 1.17145E-05 |
| Kiroba(Invitro): Mature Leaf vs Root | 130.3059153 | 12.58844369 | 0.008610417 |
| Kiroba(Invitro): Root vs Young Leaf | 246.8505769 | 9.166497171 | 0.001753711 |
| Kiroba(Invitro): Mature Leaf vs Root | 309.8511607 | 9.540059463 | 0.00035493 |
| Kiroba(Invitro): Root vs Young Leaf | 309.8511607 | 9.404537732 | 0.002453932 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 351.8970308 | 14.49704807 | 8.67375E-06 |
| Kiroba(Invitro): Root vs Young Leaf | 351.8970308 | 14.26058994 | 1.07436E-06 |
| Kiroba(Invitro): Mature | 378.5835476 | 12.58975997 | 0.00298112 |

**Table 11:** Quantitative epimarkers swhich separate different tissues for Kiroba cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kiroba(Field): Mature Leaf vs Root | 154 | 9.4738814 | 0.005436594 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 154.5674187 | 10.96222961 | 0.002714439 |
| Kiroba(Field): Mature Leaf vs Root | 157.934391 | 9.555751315 | 0.003358202 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 157.934391 | 10.76813108 | 0.002139413 |
| Kiroba(Field): Mature Leaf vs Root | 161.1483275 | 10.13039125 | 0.000527766 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 161.1483275 | 10.06605837 | 0.013037778 |
| Kiroba(Field): Mature Leaf vs Root | 245.5640164 | 10.57347977 | 0.001493162 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 245.5640164 | 10.71303489 | 0.003200879 |
| Kiroba(Field): Mature Leaf vs Root | 341.4326428 | 10.98218714 | 0.001789805 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 341.4326428 | 10.17383884 | 0.001679632 |
| Kiroba(Field): Mature Leaf vs Root | 378.5835476 | 9.191022155 | 0.008458824 |
| Kiroba(Invitro): Mature Leaf vs Root | 55.240281 | 12.65480416 | 0.012053232 |
| Kiroba(Invitro): Mature Leaf vs Root | 69.117253 | 10.48764075 | 1.51278E-12 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 69.117253 | 10.14219076 | 6.3765E-07 |
| Kiroba(Invitro): Mature Leaf vs Root | 86.680869 | 16.28063569 | 3.19406E-07 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 86.680869 | 15.84575458 | 1.17145E-05 |
| Kiroba(Invitro): Mature Leaf vs Root | 130.3059153 | 12.58844369 | 0.008610417 |
| Kiroba(Invitro): Root vs Young Leaf | 246.8505769 | 9.166497171 | 0.001753711 |
| Kiroba(Invitro): Mature Leaf vs Root | 309.8511607 | 9.540059463 | 0.00035493 |
| Kiroba(Invitro): Root vs Young Leaf | 309.8511607 | 9.404537732 | 0.002453932 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 351.8970308 | 14.49704807 | 8.67375E-06 |
| Kiroba(Invitro): Root vs Young Leaf | 351.8970308 | 14.26058994 | 1.07436E-06 |
| Kiroba(Invitro): Mature | 378.5835476 | 12.58975997 | 0.00298112 |

|  |
|---|
| Leaf vs Root |

**Table 12:** Quantitative epimarkers swhich separate different tissues for Kizimbani cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kizimbani(Field): Mature Leaf vs Root | 69.117253 | 12.32370263 | 9.43681E-06 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 69.117253 | 12.50564879 | 0.002903673 |
| Kizimbani(Field): Mature Leaf vs Root | 92.5 | 9.862881286 | 0.00764787 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 92.5 | 10.93970918 | 0.005901066 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 351.8970308 | 12.89064803 | 0.018388097 |
| Kizimbani(Field): Root vs Young Leaf | 351.8970308 | 13.0278654 | 0.005517913 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 433.5015663 | 10.22119897 | 0.013970368 |
| Kizimbani(Invitro): Root vs Young Leaf | 55.240281 | 12.92157302 | 0.001981468 |
| Kizimbani(Invitro): Mature Leaf vs Young Leaf | 69.117253 | 9.229073612 | 0.011673724 |
| Kizimbani(Invitro): Root vs Young Leaf | 69.117253 | 9.260209725 | 0.009525088 |
| Kizimbani(Invitro): Root vs Young Leaf | 133.8671134 | 11.53846926 | 0.008942668 |
| Kizimbani(Invitro): Root vs Young Leaf | 246.8505769 | 10.07289961 | 8.27362E-12 |
| Kizimbani(Invitro): Mature Leaf vs Root | 351.8970308 | 14.18371851 | 6.96135E-07 |
| Kizimbani(Invitro): Mature Leaf vs Young Leaf | 351.8970308 | 14.2497192 | 7.75834E-07 |

**Table 13:** Quantitative epimarkers swhich separate different tissues for Mfaransa cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Mfaransa(Field): Root vs Young Leaf | 351.8970308 | 15.85106677 | 0.002063959 |
| Mfaransa(Field): Root vs Young Leaf | 373.3532393 | 10.71543717 | 0.000320794 |
| Mfaransa(Invitro): Mature Leaf vs Root | 63.18 | 9.912453037 | 7.92264E-10 |
| Mfaransa(Invitro): Root vs | 63.18 | 10.39300753 | 5.95456E-15 |

| | | | |
|---|---|---|---|
| Young Leaf | | | |
| Mfaransa(Invitro): Mature Leaf vs Root | 69.117253 | 10.45190598 | 1.91982E-16 |
| Mfaransa(Invitro): Root vs Young Leaf | 69.117253 | 9.801228508 | 1.72146E-05 |
| Mfaransa(Invitro): Mature Leaf vs Root | 189.3098095 | 8.143626812 | 0.002880165 |
| Mfaransa(Invitro): Root vs Young Leaf | 189.3098095 | 8.856209565 | 0.000776321 |
| Mfaransa(Invitro): Mature Leaf vs Root | 259.3656098 | 7.778616317 | 0.0060822 |
| Mfaransa(Invitro): Root vs Young Leaf | 259.3656098 | 8.414638933 | 0.00643765 |
| Mfaransa(Invitro): Mature Leaf vs Root | 417.6775517 | 8.124851756 | 0.005157638 |

**Table 14:** Quantitative epimarkers swhich separate different tissues for Mzungu cultivar when genomic DNA was digested using *Hpa*II.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Mzungu(Field): Mature Leaf vs Root | 69.117253 | 13.47672601 | 0.0002093 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 69.117253 | 13.3056153 | 0.001496222 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 86.680869 | 10.77287137 | 0.00347176 |
| Mzungu(Field): Root vs Young Leaf | 86.680869 | 14.42051176 | 0.00585095 |
| Mzungu(Field): Mature Leaf vs Root | 92.5 | 9.578254986 | 0.002993045 |
| Mzungu(Field): Mature Leaf vs Root | 351.8970308 | 14.40522751 | 0.003508403 |
| Mzungu(Field): Mature Leaf vs Root | 378.5835476 | 9.606943175 | 0.001156628 |
| Mzungu(Invitro): Mature Leaf vs Root | 86.680869 | 15.19255466 | 2.02134E-06 |
| Mzungu(Invitro): Root vs Young Leaf | 86.680869 | 14.89822929 | 0.004239713 |
| Mzungu(Invitro): Mature Leaf vs Young Leaf | 92.5 | 10.9822332 | 6.25443E-06 |
| Mzungu(Invitro): Root vs Young Leaf | 92.5 | 11.17451629 | 4.23532E-07 |
| Mzungu(Invitro): Mature Leaf vs Root | 285.0131438 | 8.473608423 | 0.001206463 |
| Mzungu(Invitro): Mature Leaf vs Root | 309.8511607 | 9.809933307 | 3.17873E-09 |
| Mzungu(Invitro): Mature Leaf vs Young Leaf | 309.8511607 | 9.536474671 | 0.00138941 |

| | | | |
|---|---|---|---|
| Mzungu(Invitro): Mature Leaf vs Root | 351.8970308 | 13.88091058 | 1.42459E-06 |
| Mzungu(Invitro): Root vs Young Leaf | 351.8970308 | 15.00808496 | 0.005529502 |

**Table 15:** Quantitative epimarkers swhich separate different tissues for Kibandameno cultivar when genomic DNA was digested using *Msp*I.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kibandameno(Invitro): Root vs Young Leaf | 58.069093 | 17.74501975 | 0.00563216 |
| Kibandameno(Invitro): Mature Leaf vs Root | 92.5 | 10.58199476 | 4.15159E-06 |
| Kibandameno(Invitro): Root vs Young Leaf | 92.5 | 10.96866114 | 6.67368E-06 |
| Kibandameno(Invitro): Mature Leaf vs Root | 163.0598866 | 8.057027471 | 0.007943078 |
| Kibandameno(Invitro): Mature Leaf vs Root | 193.551134 | 8.548014118 | 0.001868794 |
| Kibandameno(Invitro): Mature Leaf vs Young Leaf | 263.978125 | 10.50534357 | 0.001719389 |
| Kibandameno(Invitro): Root vs Young Leaf | 263.978125 | 10.29160437 | 0.002958473 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 331.8383007 | 10.50956144 | 0.003154713 |
| Kibandameno(Invitro): Mature Leaf vs Root | 444.0787736 | 8.339969949 | 0.003045948 |
| Kibandameno(Invitro): Mature Leaf vs Root | 470.7719365 | 8.206267527 | 0.004102786 |
| Kibandameno(Invitro): Mature Leaf vs Root | 497.2937336 | 8.146780152 | 0.005325343 |
| Kibandameno(Invitro): Root vs Young Leaf | 500.2048404 | 9.141401857 | 0.00298255 |
| Kibandameno: Field vs Invitro | 55.240281 | 15.88419284 | 0.000347429 |
| Kibandameno: Field vs Invitro | 83.259582 | 16.07589412 | 8.42088E-06 |
| Kibandameno: Field vs Invitro | 130.3059153 | 14.44345938 | 0.000368766 |
| Kibandameno: Field vs Invitro | 303.9183967 | 13.65230023 | 0.000196749 |
| Kibandameno: Field vs Invitro | 316.8416961 | 13.5940129 | 0.000857588 |
| Kibandameno: Field vs Invitro | 341.4326428 | 13.84204558 | 2.09753E-10 |

| | | | |
|---|---|---|---|
| Mzungu(Invitro): Mature Leaf vs Root | 351.8970308 | 13.88091058 | 1.42459E-06 |
| Mzungu(Invitro): Root vs Young Leaf | 351.8970308 | 15.00808496 | 0.005529502 |

**Table 15:** Quantitative epimarkers swhich separate different tissues for Kibandameno cultivar when genomic DNA was digested using *Msp*I.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kibandameno(Invitro): Root vs Young Leaf | 58.069093 | 17.74501975 | 0.00563216 |
| Kibandameno(Invitro): Mature Leaf vs Root | 92.5 | 10.58199476 | 4.15159E-06 |
| Kibandameno(Invitro): Root vs Young Leaf | 92.5 | 10.96866114 | 6.67368E-06 |
| Kibandameno(Invitro): Mature Leaf vs Root | 163.0598866 | 8.057027471 | 0.007943078 |
| Kibandameno(Invitro): Mature Leaf vs Root | 193.551134 | 8.548014118 | 0.001868794 |
| Kibandameno(Invitro): Mature Leaf vs Young Leaf | 263.978125 | 10.50534357 | 0.001719389 |
| Kibandameno(Invitro): Root vs Young Leaf | 263.978125 | 10.29160437 | 0.002958473 |
| Kibandameno(Field): Mature Leaf vs Young Leaf | 331.8383007 | 10.50956144 | 0.003154713 |
| Kibandameno(Invitro): Mature Leaf vs Root | 444.0787736 | 8.339969949 | 0.003045948 |
| Kibandameno(Invitro): Mature Leaf vs Root | 470.7719365 | 8.206267527 | 0.004102786 |
| Kibandameno(Invitro): Mature Leaf vs Root | 497.2937336 | 8.146780152 | 0.005325343 |
| Kibandameno(Invitro): Root vs Young Leaf | 500.2048404 | 9.141401857 | 0.00298255 |
| Kibandameno: Field vs Invitro | 55.240281 | 15.88419284 | 0.000347429 |
| Kibandameno: Field vs Invitro | 83.259582 | 16.07589412 | 8.42088E-06 |
| Kibandameno: Field vs Invitro | 130.3059153 | 14.44345938 | 0.000368766 |
| Kibandameno: Field vs Invitro | 303.9183967 | 13.65230023 | 0.000196749 |
| Kibandameno: Field vs Invitro | 316.8416961 | 13.5940129 | 0.000857588 |
| Kibandameno: Field vs Invitro | 341.4326428 | 13.84204558 | 2.09753E-10 |

**Table 16:** Quantitative epimarkers swhich separate different tissues for Kiroba cultivar when genomic DNA was digested using *MspI*.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kiroba(Invitro): Mature Leaf vs Root | 55.240281 | 13.64418894 | 2.34889E-10 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 66.9356 | 10.04986395 | 0.01175502 |
| Kiroba(Field): Root vs Young Leaf | 76.843673 | 9.578972084 | 2.59278E-08 |
| Kiroba(Field): Mature Leaf vs Root | 193.551134 | 8.526272984 | 0.011275176 |
| Kiroba(Field): Root vs Young Leaf | 346.4151791 | 12.63090099 | 2.95505E-05 |
| Kiroba(Field): Root vs Young Leaf | 348.770263 | 12.63530656 | 2.54059E-05 |
| Kiroba(Field): Root vs Young Leaf | 442.3945991 | 8.61812883 | 0.003217131 |
| Kiroba(Field): Mature Leaf vs Root | 500.2048404 | 9.793083664 | 4.76926E-05 |
| Kiroba(Field): Mature Leaf vs Young Leaf | 500.2048404 | 8.794233593 | 0.010167567 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 55.240281 | 13.27840321 | 0.014774959 |
| Kiroba(Invitro): Mature Leaf vs Root | 76.843673 | 10.10231479 | 6.73822E-12 |
| Kiroba(Invitro): Root vs Young Leaf | 76.843673 | 10.38424817 | 1.49481E-05 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 97.7 | 9.855512356 | 0.000240567 |
| Kiroba(Invitro): Root vs Young Leaf | 97.7 | 9.826661059 | 0.000129634 |
| Kiroba(Invitro): Mature Leaf vs Root | 263.978125 | 9.515364698 | 0.007994861 |
| Kiroba(Invitro): Root vs Young Leaf | 263.978125 | 9.452645605 | 0.010894014 |
| Kiroba(Invitro): Mature Leaf vs Root | 266.8722222 | 13.80432152 | 6.66928E-06 |
| Kiroba(Invitro): Root vs Young Leaf | 266.8722222 | 13.54652732 | 0.011836007 |
| Kiroba(Invitro): Mature Leaf vs Young Leaf | 346.4151791 | 12.92572532 | 0.005306922 |
| Kiroba(Invitro): Mature Leaf vs Root | 458.9518627 | 8.55137908 | 0.012192333 |
| Kiroba(Invitro): Root vs Young Leaf | 500.2048404 | 9.118273003 | 0.001259835 |

**Table 17:** Quantitative epimarkers which separate different tissues for Kizimbani cultivar when genomic DNA was digested using *Msp*I.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Kizimbani(Field): Mature Leaf vs Root | 76.843673 | 9.241037787 | 0.000600306 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 76.843673 | 10.74313264 | 8.53279E-05 |
| Kizimbani(Field): Mature Leaf vs Root | 113.16412 | 8.294572605 | 0.001419666 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 137.392432 | 9.917075639 | 0.001793974 |
| Kizimbani(Field): Root vs Young Leaf | 137.392432 | 10.21759298 | 0.000497324 |
| Kizimbani(Field): Mature Leaf vs Root | 193.551134 | 9.227350085 | 0.00055951 |
| Kizimbani(Field): Root vs Young Leaf | 193.551134 | 9.647846339 | 0.002522163 |
| Kizimbani(Field): Mature Leaf vs Root | 220.1480556 | 8.303315011 | 0.000756876 |
| Kizimbani(Field): Mature Leaf vs Root | 243.408141 | 8.261580086 | 0.001913534 |
| Kizimbani(Field): Root vs Young Leaf | 243.408141 | 8.682594535 | 0.003011523 |
| Kizimbani(Field): Mature Leaf vs Root | 259.3656098 | 8.892847653 | 0.001481866 |
| Kizimbani(Field): Mature Leaf vs Young Leaf | 263.978125 | 11.80519035 | 4.51996E-07 |
| Kizimbani(Field): Root vs Young Leaf | 309.8511607 | 8.813589009 | 0.002982491 |
| Kizimbani(Field): Mature Leaf vs Root | 319.8398899 | 8.798814032 | 0.011854125 |
| Kizimbani(Field): Mature Leaf vs Root | 370.935 | 8.080790821 | 0.00218095 |
| Kizimbani(Field): Mature Leaf vs Root | 417.6775517 | 7.945253093 | 0.003918345 |
| Kizimbani(Field): Mature Leaf vs Root | 475.8546602 | 8.472314863 | 0.000801303 |
| Kizimbani(Field): Mature Leaf vs Root | 482.1002 | 8.056561379 | 0.002696807 |
| Kizimbani(Field): Root vs Young Leaf | 488.2717391 | 9.011878844 | 0.007095615 |
| Kizimbani(Field): Mature Leaf vs Root | 497.2937336 | 7.912637096 | 0.003902784 |
| Kizimbani(Field): Mature Leaf vs Root | 500.2048404 | 9.850862109 | 4.52392E-05 |
| Kizimbani(Field): Root | 500.2048404 | 10.27384372 | 0.000419421 |

| | | | |
|---|---|---|---|
| vs Young Leaf | | | |
| Kizimbani(Field): Mature Leaf vs Root | 502.7 | 8.052036698 | 0.002608886 |
| Kizimbani(Field): Mature Leaf vs Root | 521.1826282 | 8.431099011 | 0.001002284 |
| Kizimbani(Field): Root vs Young Leaf | 521.1826282 | 8.849239146 | 0.004671173 |
| Kizimbani(Field): Mature Leaf vs Root | 526.0944737 | 8.264853704 | 0.002601028 |
| Kizimbani(Field): Root vs Young Leaf | 526.0944737 | 8.68593708 | 0.002552993 |
| Kizimbani(Invitro): Mature Leaf vs Root | 101.1418059 | 14.59205986 | 0.014084985 |

**Figure 18:** Quantitative epimarkers swhich separate different tissues for Mfaransa cultivar when genomic DNA was digested using *Msp*I.

| Comparison | Epilocus | logCPM | PValue |
|---|---|---|---|
| Mfaransa(Field): Root vs Young Leaf | 92.5 | 13.31807164 | 6.00451E-08 |
| Mfaransa(Field): Root vs Young Leaf | 109.5816011 | 9.84296076 | 0.006037582 |
| Mfaransa(Field): Root vs Young Leaf | 245.5640164 | 9.909488596 | 0.006194229 |
| Mfaransa(Field): Root vs Young Leaf | 332.8671429 | 12.07797242 | 0.000148557 |
| Mfaransa(Field): Root vs Young Leaf | 341.4326428 | 9.402011894 | 0.013285174 |
| Mfaransa(Field): Root vs Young Leaf | 348.770263 | 14.65784918 | 0.003884877 |
| Mfaransa(Field): Root vs Young Leaf | 457.7534591 | 9.929477931 | 0.000910313 |
| Mfaransa(Invitro): Mature Leaf vs Root | 55.240281 | 13.72777267 | 2.29391E-06 |
| Mfaransa(Invitro): Mature Leaf vs Root | 76.843673 | 9.779674847 | 9.90928E-08 |
| Mfaransa(Invitro): Root vs Young Leaf | 76.843673 | 9.225141855 | 0.001759603 |
| Mfaransa(Invitro): Mature Leaf vs Root | 92.5 | 9.288462021 | 0.013124187 |
| Mfaransa(Invitro): Mature Leaf vs Young Leaf | 92.5 | 9.459354828 | 0.004798679 |
| Mfaransa(Invitro): Mature Leaf vs Root | 309.8511607 | 9.327485506 | 0.00097062 |
| Mfaransa(Invitro): Mature Leaf vs Young Leaf | 309.8511607 | 9.300013086 | 0.004539962 |
| Mfaransa(Invitro): | 417.6775517 | 8.604439402 | 0.010759225 |

| | | | |
|---|---|---|---|
| Mature Leaf vs Root | | | |
| Mfaransa(Invitro): Mature Leaf vs Young Leaf | 500.2048404 | 8.24142981 | 0.013677173 |
| Mfaransa(Invitro): Mature Leaf vs Root | 521.1826282 | 8.774728129 | 0.006103134 |

**Figure 18:** Quantitative epimarkers swhich separate different tissues for Mzungu cultivar when genomic DNA was digested using *MspI*.

| Comparison | Epilocus | logCPM | P-Value |
|---|---|---|---|
| Mzungu(Field): Mature Leaf vs Young Leaf | 69.117253 | 12.88971497 | 8.91885E-18 |
| Mzungu(Field): Root vs Young Leaf | 69.117253 | 10.14947233 | 0.001558132 |
| Mzungu(Field): Mature Leaf vs Root | 97.7 | 10.25999491 | 1.27866E-10 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 97.7 | 10.17129328 | 5.90867E-06 |
| Mzungu(Field): Root vs Young Leaf | 161.1483275 | 8.922417218 | 0.002523913 |
| Mzungu(Field): Root vs Young Leaf | 189.3098095 | 8.140645439 | 0.006529274 |
| Mzungu(Field): Root vs Young Leaf | 290.6145989 | 7.876969292 | 0.009336376 |
| Mzungu(Field): Mature Leaf vs Root | 308.3116563 | 8.952955585 | 0.001774281 |
| Mzungu(Field): Mature Leaf vs Root | 309.8511607 | 9.315414349 | 0.002101316 |
| Mzungu(Field): Mature Leaf vs Root | 311.1962252 | 9.166773581 | 0.011092252 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 346.4151791 | 13.80880699 | 3.03746E-08 |
| Mzungu(Field): Root vs Young Leaf | 346.4151791 | 14.12938243 | 1.05164E-06 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 348.770263 | 13.83268241 | 2.85421E-08 |
| Mzungu(Field): Root vs Young Leaf | 348.770263 | 14.14781784 | 0.000885734 |
| Mzungu(Field): Mature Leaf vs Young Leaf | 444.0787736 | 8.494462938 | 0.006230139 |
| Mzungu(Field): Root vs Young Leaf | 444.0787736 | 9.009590052 | 0.002495352 |
| Mzungu(Field): Root vs Young Leaf | 500.2048404 | 8.841001188 | 0.002687417 |
| Mzungu(Field): Mature Leaf vs Root | 521.1826282 | 9.434536081 | 3.39238E-08 |
| Mzungu(Field): Root vs Young Leaf | 521.1826282 | 9.389081392 | 0.000766176 |
| Mzungu(Invitro): | 55.240281 | 14.15665353 | 0.000740952 |

| | | | |
|---|---|---|---|
| Mature Leaf vs Root | | | |
| Mzungu(Invitro): Mature Leaf vs Root | 161.1483275 | 8.626833276 | 0.002657339 |
| Mzungu(Invitro): Mature Leaf vs Root | 163.0598866 | 8.653287984 | 0.001869582 |
| Mzungu(Invitro): Mature Leaf vs Root | 225.8361468 | 8.613349449 | 0.003055834 |
| Mzungu(Invitro): Mature Leaf vs Root | 444.0787736 | 8.837621975 | 0.001780721 |
| Mzungu(Invitro): Mature Leaf vs Root | 458.9518627 | 7.973833675 | 0.01179232 |
| Mzungu(Invitro): Mature Leaf vs Root | 500.2048404 | 9.542904722 | 1.00464E-05 |
| Mzungu(Invitro): Mature Leaf vs Root | 510.6046023 | 8.514067392 | 0.006921242 |
| Mzungu(Invitro): Mature Leaf vs Young Leaf | 518.9734951 | 9.021039441 | 0.011303029 |