Common garden experiment reveals altered nutritional values and DNA methylation profiles in micropropagated three elite Ghanaian sweet potato genotypes

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Abstract

Micronutrient deficiency is the cause of multiple diseases in developing countries. Staple crop biofortification is an efficient means to combat such deficiencies in the diets of local consumers. Biofortified lines of sweet potato (*Ipomoea batata* L. Lam) with enhanced beta-carotene content have been developed in Ghana to alleviate Vitamin A Deficiency. These genotypes are propagated using meristem micropropagation to ensure the generation of virus-free propagules. *In vitro* culture exposes micropropagated plants to conditions that can lead to the accumulation of somaclonal

variation with the potential to generate unwanted aberrant phenotypes. However, the effect of micropropagation induced somaclonal variation on the production of key nutrients by field-grown plants has not been previously studied. Here we assessed the extent of *in vitro* culture induced somaclonal variation, at a phenotypic, compositional and genetic/epigenetic level, by comparing field-maintained and micropropagated lines of three elite Ghanaian sweet potato genotypes grown in a common garden. Although micropropagated plants presented no observable morphological abnormalities compared to field maintained lines, they presented significantly lower levels of iron, total protein, zinc, and glucose. Methylation Sensitive Amplification Polymorphism analysis showed a high level of *in vitro* culture induced molecular variation in micropropagated plants. Epigenetic, rather than genetic variation, accounts for most of the observed molecular variability. Taken collectively, our results highlight the importance of ensuring the clonal fidelity of the micropropagated biofortified lines in order to reduce potential losses in the nutritional value prior to their commercial release.

Introduction

Sweet potato (*Ipomoea batatas* L. Lam), is a drought tolerant, low input, and high yielding crop, which produces more nutrients and has higher edible energy than most staples such as rice, cassava, wheat, and sorghum [1]. As a predominantly vegetatively propagated crop, virus accumulation in vegetative propagules (i.e. vine cuttings and tubers) can cause devastating loss in yield and poor root quality in subsequent cultivation [2]. Micropropagation techniques, such as meristem or nodal tip culture, coupled with thermotherapy or cryotherapy, are currently the principal plant tissue culture (PTC) methods for producing healthy (pathogen-tested/disease-free) clones of planting materials [3]. However, the generation of true-to-type material through *in vitro* propagation can be challenging due to somaclonal variation [4].

Somaclonal variation refers to changes that can be induced during *in vitro* tissue culture and have been reported in all *in vitro* systems [5-8]. Such changes can be genetic and/or epigenetic in nature. Epigenetic modifications are heritable changes that can affect the phenotype without changes to the DNA sequence [9]. These are mediated, among other mechanisms, by DNA methylation, small RNA mediated silencing, histone modification, and chromatin remodelling [10]. DNA methylation is the addition of a methyl group to carbon 5 in the pyrimidine ring of cytosines [11]. In plants, DNA methylation occurs at the CG, CNN, or CNG context (where N=C, A, or T), and has been shown to induce changes in gene expression, which has the potential to lead to phenotypic changes [12]. Environmental conditions can induce changes to plant methylomes [13, 14]. *In vitro* culture of plant tissues has been reported to induced epigenetic somaclonal variation for multiple crop species including garlic [15], cassava [6], pineapple [16], cotton [17], cocoa [7], and other crops [5, 18]. However, few studies have evaluated the extent of DNA methylation changes during

60 meristem propagation of sweetpotato. In addition, no study has been conducted to understand the
61 correlation between the extent of *in vitro* induced epigenetic changes and the nutritional
62 composition of sweet potato tubers.
63
64 Vitamin A is an essential nutrient that is required in small amounts for maintaining healthy growth
65 and development, particularly in growing children, pregnant and lactating mothers [19]. Vitamin
66 A deficiency (VAD) has been declared a public health problem affecting up to 48% of children in
67 sub-Saharan African countries including Ghana [20]. VAD manifests itself as severe respiratory
68 infections, diarrhoeal diseases and eye diseases ranging from night blindness, to the more serious
69 sight condition, keratomalacia (melting of the cornea) and even mortality [21]. Beta carotene is a
70 precursor to Vitamin A abundant in plant cells [22]. Biofortification is an affordable tool to combat
71 nutrient deficiencies and hence sweet potato is currently being biofortified for enhanced beta
72 carotene content to combat micronutrient malnutrition. It is, however, crucial to understand the
73 impact that somaclonal variation via micropropagation has on the nutritional content of biofortified
74 sweet potato tubers. Thus, our aim was to test the hypothesis that the nutritional value of
75 micropropagated plants could be affected by somalconal variation. To achieve this we assesed
76 differences in plant morphology and nutritional composition of *in vitro* and field-maintained
77 propagules of three improved sweet potato genotypes, while evaluating the incidence of molecular
78 (genetic and epigenetic) somaclonal variation. Micropropagated and field maintained plants of the
79 selected sweet potato genotypes were grown in a common garden experiment and examined for
80 phenotypic variation in the micropropagated regenerants. Near Infrared Spectrophotometry
81 (NIRS) [23] was used to analyse the nutritional composition in mature tubers from both types of
82 propagules. Finally, Methylation Sensitive Amplification Polymorphism (MSAP), which is a

rapid, cost effective, and reliable method of assessing epigenetic variability [24], was used to investigate the extent of genetic and epigenetic variability imposed by *in vitro* culture on micropropagated plants.

Materials and methods

Field experimental design

Field work for this study was conducted during the major growing season (March-July, 2016), at the Council for Scientific and Industrial Research-Crops Research Institute (CSIR-CRI), Fumesua, located in the Forest agro-ecological zone of Ghana (N 6.43'25º, W 1.31'9º). The land was cleared, ploughed, ridged, and manured with poultry droppings [25]. Three CRI improved genotypes with moderate resistance to sweet potato viruses were used for this study: CRI-Bohye, CRI-Ogyefo, and CRI-Otoo (Table S1). Micropropagated clones of these genotypes, produced by meristem-tip culture and thermotherapy and maintained for 18 months in Plant Tissue Culture [3, 26], were obtained from the screen house. Cuttings from visually virus free planting vines of the same genotypes maintained according to the agronomic practices of the institute on the CSIR-CRI multiplication field (field-maintained), since year of release (Table S1), were also obtained. Both types of propagules were planted in a Randomized Complete Block Design with three replicated blocks (Fig. S1). Eight plants per plot were randomly selected for molecular, morphological and nutritional analysis.

Phenotypic characterisation

To examine the incidence of phenotypic somaclonal variation, all plants were scored on a scale of 1-9 for selected foliage and storage roots characteristics based on standard sweet potato morphological descriptors [27] (Table S2). Selected descriptors were based on pigmentation in the leaves, roots, and vines. The foliage parameters were scored between 90-100 days after planting, and they included immature and mature leaf colour, abaxial leaf vein pigmentation, predominant vine colour, secondary vine colour, petiole pigmentation, and plant type. Storage root descriptors, i.e. shape, predominant skin colour, and flesh colour were documented at 120 days after planting [27].

Nutritional analysis

To study the tissue culture induced changes to the composition of mature tubers, nutritient content analysis of the storage roots was done at harvest. Analysis was carried out at the Quality and Nutritional laboratory, CSIR-CRI. In brief, harvested roots were pooled by block/genotype/propagation system (n=3). Each pool was then sampled individually as described by Amankwaah [28]. Tubers were then washed, air dried, peeled, quartered, sliced, weighed (50g), and freeze dried for 73 hours using a YK-118 Vacuum Freeze Dryer (True Ten Industrial Company Limited Taichung, Taiwan). Freeze-dried weights were recorded, and dry matter was computed based on the differences between the fresh and freeze-dried weights as: Percentage dry matter = dry weight/fresh weight × 100. Freeze-dried tuber samples were then milled (3383-L70, Thomas Scientific, Dayton Electric Manufacturing Company Limited, IL 60714, USA), and analysed using Near Infrared Spectrophotometry (NIRS) (XDS Rapid Content Analyzer, Hoganae, Sweden) to

estimate starch (%), protein (%), zinc (mg $100g^{-1}$), fructose (%), glucose (%), iron (mg $100g^{-1}$) and sucrose (%) content [29].

Analysis of plant DNA methylation profiles

DNA extraction

In all, 144 plants were sampled for DNA extraction, comprising of 48 samples for each of the three genotypes (24 micropropagated, 24 field-maintained). The youngest leaves of 6 weeks old plants were collected on ice from the field, and kept in liquid nitrogen until DNA extraction with the modified CTAB protocol [30]. Agarose gel electrophoresis (0.8%) was used to check the quality of the extracted DNA, while the concentration and purity were analysed using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA was diluted to 20 ng μl^{-1} for MSAP analysis.

Methylation Sensitive Amplification Polymorphism (MSAP) Profiling

To investigate the tissue culture induced changes to cytosine methylation, MSAP was performed based on established protocol [31]. Genomic DNA was digested with a combination of one of two methylation sensitive isoschizomers as frequent cutter (i.e. *Hpa*II or *Msp*I which present the same recognition sequence (CCGG), but different sensitivity to DNA methylation), and the methylation insensitive *Eco*RI as rare cutter, which has the recognition site GAATTC. Restriction products were then ligated to double stranded DNA adaptors with co-adhesive ends complementary to those present in *Hpa*II/*Msp*I and *Eco*RI restriction products using T4 DNA ligase. Pre-selective amplification was then done using primers complimentary to the adaptor sequence, but with unique

3'overhangs (Table S3). A second round of selective amplification was then carried out using primers with extra selective bases and labelled with a 6-FAM reporter molecule for fragment detection (Table S3). Finally, amplified products were capillary electrophoresed using the ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Australian Genome Research Facility Ltd, Adelaide, South Australia. MSAP capillary electrophoresis profiles were transformed into a presence (1) or absence (0) binary matrix for both *Hpa*II/*Eco*R1 and *Msp*I/*Eco*R1 restriction products, using GeneMapper Software v4 (Applied Biosystems, Foster City, CA).

To identify the most informative MSAP primer combinations for this study, twelve selective primer combinations (Table S4) were tested on three micropropagated and three field-maintained DNA samples from one genotype (Bohye). One of the three samples of each group was duplicated to assess marker reproducibility for each primer combination. Primer reproducibility (i.e. % of MSAP markers present in both replicates), number of alleles, number of differential alleles (i.e. number of polymorphic markers between field maintained and micropropagated samples), and principal coordinate analysis (PCoA) of MSAP profiles of all the primer combinations were analysed to determine the two most informative primer pairs that were then used in all samples.

Statistical analysis

To examine phenotypic, nutrient content, and virus incidence data, GenStat (15th Edition) was used to perform Analysis of Variance (ANOVA), and to statistically test any detected difference between means at 5% least significant difference (LSD).

168

169 GenAlEx v6.5 [32] and R package *msap* v.3.3.1 [24] were used to investigate the level of tissue
170 culture induced genetic and epigenetic variation by analysing both types of variability among and
171 between field-maintained and micropropagated samples for each of the 3 genotypes tested as
172 described previously [13, 31]. In brief, first we used Principal Coordinate Analysis (PCoA) in
173 GenAlEx v6.5 to visualise the molecular diversity (genetic and epigenetic) captured by MSAP
174 profiles generated with each primer (E and I) and enzyme combination (*Hpa*II/*EcoR*I and *Msp*I
175 *EcoR*I) individually. Then Analysis of Molecular Variance (AMOVA) was computed on GenAlEx
176 v6.5 to estimate pairwise molecular distances (PhiPT) between the field-maintained and
177 micropropagated plant populations. The significance of the observed PhiPT values was assigned
178 by random permutations tests (based on 9,999 replicates).

179

180 To determine to what extent the observed changes in MSAP profiles could be attributed to genetic
181 (changes in DNA sequence) or epigenetic (changes in the DNA methylation patterns) of the
182 studied samples, we identified Non-Methylated Loci (NML) and Methylation Susceptible Loci
183 (MSL) by comparing the MSAP profiles generated using *Hpa*II/*EcoR*I and *Msp*I *EcoR*I as
184 implemented in *msap v.3.3.1*. First, Principal Component Analysis (PCA) was used to visualise
185 the contribution of each type of change, then Shannon diversity Index (S) and Wilcoxon Rank Sum
186 test were used to estimate the contribution and statistical significance of each type of variability
187 (genetic and epigenetic).

188

Results

Morphological characterisation

No significant variation was observed for any of the 8 phenotypic traits measured i.e. foliage colour (for immature and mature plants), plant type, petiole pigmentation, abaxial leaf vein pigmentation, storage root shape, storage root pigmentation, and flesh colour between micropropagated and field-maintained plants in any of the genotypes analysed (Table S5).

Nutritional composition

Analysis of variance of eight nutrients in mature sweet potato tubers showed that iron, total protein, zinc, and glucose levels were significantly higher ($P<0.05$) in field-maintained than in micropropagated sweet potato plants (Fig 1, Table 1). Sucrose, fructose, dry matter, and starch contents were not significantly different between micropropagated and field propagules for the genotypes analysed (Table 1). For all measured traits (excluding sucrose and zinc), variability was higher in micropropagated samples than in field maintained ones (Fig 1, Table 1).

Fig 1. Effect of somaclonal variation on nutritional composition of sweet potato tubers. Box and whisker plot showing the content of glucose, iron, protein, and zinc in *in vitro* and field-maintained sweet potato tubers. Micropropagated and field maintained plants from three genotypes (Bohye, Ogyefo, and Otoo) were grown on a Randomized Complete Block Design with three replicated blocks and 24 plants per block/genotype/propagation system. Plants from each

209 block/genotype/propagation system were pooled and analysed using Near Infrared
210 Spectrophotometry.

211

212 **Table 1. Compositional analysis of mature tubers from field maintained (Field) and**
213 **micropropagated (*in vitro*) sweet potato plants**. Values show the mean values and standard
214 deviation (in parenthesis) of nutrients in sweet potato tubers from three genotypes
215 micropropagated and field maintained. Three replicate measurements were taken for each
216 genotype/propagation system combination (n = 9). l.s.d.: least significant difference; n.s.: not
217 significant; * P<0.05; ** P<0.005.

Prop system	Dry Matter %	Fructose %	Glucose %	Iron mg/100g	Protein %	Starch %	Sucrose %	Zinc mg/100g
Field	30.64 (2.37)	1.32 (0.31)	2.74 (0.31)	2.46 (0.16)	5.95 (1.02)	67.45 (1.16)	5.61 (4.60)	1.31 (0.21)
In vitro	31.96 (3.46)	1.38 (0.47)	2.36 (0.55)	1.91 (0.37)	4.68 (1.39)	69.56 (4.21)	4.71 (2.06)	0.98 (0.20)
l.s.d (5%)	n.s.	n.s.	0.17**	0.26**	1.10*	n. s	n. s	0.20*

218

219 # Assessment of micropropagation induced molecular variability

220 We first used a reduced number of samples to test 12 MSAP primer combinations to identify the
221 most informative and reproducible primer combinations. These generated between 149 and 205
222 loci (Combinations K and D respectively) (Table S4). Estimated loci reproducibility ranged from
223 91 to 98% (Combinations G and L) (Table S4). The number of loci discriminating between field-
224 maintained and micropropagated samples varied between 1 and 12 (Combinations H and C
225 respectively) (Table S4). After comparison of the principal coordinates analysis results (Fig. S2),

226 number of alleles, reproducibility, and number of discriminatory alleles between *in vitro* and field
227 maintained plants for each of the 12 primer combinations, primer combinations E and I (Table S4)
228 were selected to analyse differences between the entire sample set of *in vitro* and field maintained
229 plants. These produced 197 and 174 alleles, 9 and 11 discriminatory alleles, and a reproducibility
230 of 98% and 93% respectively.

231

232 When applied to all samples, primer combinations E and I generated a total of 244 and 235 loci
233 respectively. PCoA and PCA of MSAP profiles containing all 479 loci showed that *in vitro*
234 maintained samples, irrespective of their genotype, shift in the same direction, e.g. MSAP profiles
235 generated from micropropagated plants, using both *Hpa*II and *Msp*I and irrespective of their
236 background genotype, are displaced towards the top quadrants in relation to the field maintained
237 plants, when analysed with GenAlex v6.5 (Fig. S3a) or towards the right quadrants when analysed
238 using *msap* v.3.3.1 (Fig. S3b).

239 PCoA (as implemented on GenAlex v6.5) of MSAP profiles from samples grouped by genotype,
240 showed clear separation between the populations of micropropagated and field maintained samples
241 for each of the three genotypes studied (Fig 2). Analysis of the Molecular Variance (AMOVA)
242 showed that differences between micropropagated and field maintained plants explain 13, 27 and
243 29% and 7, 22, and 24% of total variability observed for Ogyefo, Otoo, and Bohye for *Msp*I and
244 *Hpa*II restriction products respectively. Pairwise molecular distances (PhiPT) calculated between
245 micropropagated and field-maintained plant populations showed that all genotype/enzyme/primer
246 combinations generated significant differences (P value < 0.005). Of these, micropropagated and
247 field-maintained Ogyefo plants showed lower levels of molecular differentiation than those shown
248 by Bohye and Otoo plants (Table 2). In all genotype/enzyme/primer combinations,

micropropagated samples occupied a much larger Eigen space than their field-maintained counterparts (Fig 2).

Fig 2. Analysis of somaclonal variation induced by micropropagation in three sweet potato genotypes. PCoA generated using GenAlex v6.5 from MSAP profiles from 144 micropropagated (empty symbols) and field-maintained (full symbols) plants from genotypes of Bohye (green), Ogyefo (red), and Otoo (blue) (n = 24). MSAP profiles were amplified from genomic DNA restricted using *Hpa*II (a1-f1) and *Msp*I (a2-f2) and amplified using primer combinations E (a-c) and I (d-f). Plants were grown on a Randomized Complete Block Design with three replicated blocks and 24 plants/block/genotype/propagation system.

Table 2. Analysis of molecular differentiation between field maintained and micropropagated sweet potato plants. Molecular distance (PhiPT) was calculated as implemented in GenAlex v6.5 using MSAP profiles generated from DNA from field maintained and micropropagated plants of three sweet potato genotypes (Bohye, Ogyefo, and Otoo) (n=24) restricted using *Hpa*II and *Msp*I and amplified using primer I and E. P values were calculated by random permutations tests based on 9,999 replicates.

	Primer combination I				*Primer combination E*			
	*Msp*I		*Hpa*II		*Hpa*II		*Msp*I	
Genotypes	PhiPT	P-value	PhiPT	P-value	PhiPT	P-value	PhiPT	P-value
Bohye F/M	0.289	0.0001	0.218	0.001	0.168	0.0010	0.264	0.001
Ogyefo F/M	0.133	0.0001	0.068	0.002	0.088	0.0001	0.106	0.001

Otoo F/M	0.266	0.0001	0.241	0.001	0.166	0.0001	0.196	0.001

We then used *msap* v.3.3.1 R package to determine the contribution of epigenetic (Methylation-Susceptible Loci (MSL)) and of genetic (Non-Methylated Loci (NML)) to *in vitro* culture induced variability. PCA analysis of MSL and NML generated using both primer combinations showed separation between field-maintained and micropropagated plants (Fig S4). Pairwise PhiST distances between *in vitro* culture and field maintained plants showed epigenetic distances (i.e. PhiST calculated using MSL) were higher than those calculated using NML (i.e. genetic PhiST) for all genotypes (Table 3). As seen with PhiPT values, pairwise PhiST distances calculated for MSL and NML revealed that Ogyefo recorded the lowest epigenetic and genetic distances between micropropagated and field maintained plants, while Bohye presented the highest epigenetic distance, and Otoo had the highest genetic distance (Table 3). Shannon diversity Index (S) performed for MSL were higher (Paired sample T-test Pval<0.0001) than those for NML (Table 3). Wilcoxon Rank Sum test with continuity correction, which tests the significance of the Shannon Index, confirmed that the SIs calculated for MSL were statisticaly significant ($P < 0.0001$) while those calculated for NML were not (Table 3).

Table 3. Contribution of epigenetic and genetic polymorphisms to the molecular differentiation in sweet potato plants. MSL and NML were identified implementing msap package in R to MSAP profiles generated from DNA from field maintained and micropropagated plants of three sweet potato genotypes (Bohye, Ogyefo, and Otoo) (n=24) restricted using *Hpa*II

287 and *Msp*I and amplified using primer E and I. #: number of loci; SI: Shannon Index; NA: not
288 applicable; †Wilcoxon rank sum test with continuity correction showing P < 0.0001; * P<0.0001.

Primer combination E

	Methylation-Susceptible Loci (MSL)				No Methylated Loci (NML)			
	[1]#	PhiST	P-value	SI	#	PhiST	P-val	SI
All genotypes	181	[4]NA	0.0001*	0.50469†	63	NA	0.0001*	0.23183
Bohye	172	0.113	0.0001*	0.47136†	72	0.107	0.0001*	0.20482
Ogyefo	152	0.085	0.0001*	0.50486†	92	0.027	0.0595	0.25226
Otoo	151	0.097	0.0001*	0.52322†	93	0.115	0.0001*	0.25464

Primer combination I

	Methylation-Susceptible Loci				No Methylated Loci			
	#	PhiST	P-val	SI	#	PhiST	P-val	SI
All genotypes	171	NA	0.0001*	0.5082†	64	NA	0.0001*	0.2098
Bohye F/M	153	0.235	0.0001*	0.4939†	82	0.052	0.0009*	0.2048
Ogyefo F/M	145	0.130	0.0001*	0.4998†	90	0.039	0.0029*	0.2333
Otoo F/M	152	0.135	0.0001*	0.5131†	83	0.179	0.0001*	0.2290

Analysis of differences in band presence/absence between samples restricted with *Hpa*II or *Msp*I was used to infer the contribution of each type of methylation present on the enzymes recognition site (i.e CCGG). When considering all samples collectively (Fig 3), fully methylated recognition sites (i.e. all cytosines methylated) represented the majority of the analysed loci (48.2%), followed by fully unmethylated sites (21.5%), hemimethylated sites (i.e. only one DNA strand methylated presenting cytosines) (19.3%), and sites presenting internal cytosine methylation (11.2%). DNA from micropropagated plants presented lower levels of unmethylated (19.1 vs 23.9%) and hemimethylated sites (18.1 vs 20.5%) . Micropropagated plants also showed higher levels of fully methylated sites (49.4 vs 47.1%) and of internal cytosine methylation (13.4 vs 9.04%) (Fig 3). Individual analysis by genotype revealed that Ogyefo propagules presented lowest levels on differentiation between micropropagated and field maintained plants (Fig. S4).

Figure 3. Analysis of somaclonal variation in sweet potato. Percentage of unmethylated loci (empty bars), hemimethylated loci (bars with diagonal pattern), loci containing internal methylation (dotted bars) and fully methylated loci (black bars) on MSAP profiles generated from micropropagated (VF) and field samples (FM) (n=24) from three sweet potato genotypes (Bohye, Ogyefo, Otoo) as determined by *msap* package in R.

Discussion

The passage of plant tissues through *in vitro* culture may induce undesired variability in the regenerated propagules called somaclonal variation [10]. However, in some cases, *in vitro* culture

310 is indispensable for the production and multiplication of disease-free planting materials in
311 vegetatively propagated crops like sweet potato [33]. To test to what extent *in vitro* culture induced
312 somaclonal variation could affect the nutritional value of micropropagated sweet potatoes, we
313 compared field maintained morphological, chemical (nutritional composition) and molecular
314 (genetic and epigenetic) variation in micropropagated sweet potato mericlones.

315

316 Micropropagation alters sweet potato nutritional value

317 Phenotypic characterization, based on standard sweet potato morphological descriptors of
318 micropropagated regenerants and their field-maintained counterparts, grown on a common garden
319 setup, did not show any significant differences between both types of propagules. Conversely,
320 compositional comparative analysis of mature tubers showed that 4 out of the 8 nutritional traits
321 analysed (i.e. iron, zinc, total protein, and glucose) were significantly lower in micropropagated
322 plants compared to field-maintained individuals. Also, the variability between samples in 6 out of
323 the 8 measured nutrients (dry matter, fructose, glucose, iron, total protein, and starch), was higher
324 in micropropagated plants than in field maintained plants. Previous studies have shown that the
325 components of the growth media that are supplemented to tissue culture plants can have an effect
326 on *in vitro* plants [6]. In this study, however, both micropropagated and field-maintained plants
327 were grown in the same conditions from planting until harvest (four months). This indicates that
328 the detected differences in tuber composition could be associated to somaclonal variability induced
329 during culture that is maintained after plant establishment in the field. Both genetic and epigenetic
330 somaclonal variability can be faithfully maintained during multiple cell divisions [6, 7], and
331 therefore could be the source of the tuber compositional differences observed here.

Micropropagation induces somaclonal variation in sweet potato

To determine the level and nature of somaclonal variation induced by micropropagation we analysed the MSAP profiles of 144 plants (i.e. 48 samples for each of the three genotypes (24 micropropagated, 24 field-maintained)), grown in a common garden set up. Multivariate analysis (PCoA and PCA) revealed that MSAP profiles of micropropagated plants are different from those of generated from field maintained plants. Analysis of Molecular Variance (AMOVA) showed that between 7 and 29% of the total observed molecular variability can be explained by the influence of *in vitro* culture conditions on micropropagated samples. Estimation of the molecular distance (PhiPT) between both types of propagules showed that the observed separation was significant for all genotypes. Although micropropagation is generally considered to induce low levels of somaclonal variation, our results are in concordance with previous studies in multiple species, e.g. cassava [6], grapevine [34], hop [35], tomato [36], triticale [37], and wild barley [38]. PCoA and PCA also revealed a higher level of variability within micropropagated samples compared to field maintained samples, which supports the somaclonal origin of the observed variability. The observed high level of variability within micropropagated sweet potato ramets, would suggest that a high proportion of the detected variability is random in nature. According to Smulders and De Klerk [39], the reason for these random changes might be attributable to the extreme conditions exposed to tissue culture plants such as abnormal photoperiods, wounding, application of growth regulators, among others. These may lead to oxidative stress, which can cause epigenetic or genetic changes to the genome, leading to somaclonal variants [40]. Interestingly, our results also show that the variability acquired by micropropagated ramets from all three genotypes occupied similar Eigen space in relation to their field maintained counterparts.

355 This indicates that the observed somaclonal variability is not entirely random as previously seen
356 in other species e.g. cocoa [7, 8].
357

358 Somaclonal variation is mainly driven by DNA methylation
359 polymorphisms

360 To determine the nature (genetic or epigenetic) of the detected somaclonal variation, we first
361 identified the number of methylation sensitive and of non-methylated loci (MSL and NML
362 respectively) using the *msap* v.3.3.1 R package. Between 62 and 70% (depending on the
363 genotype/primer combination analysed) of all analysed loci were MSL. This level of methylation
364 sits within the range of those previously described for plant species [41] for the CG and CNG
365 contexts present within the recognition site of *Hpa*II and *Msp*I. PCA analysis of MSL and NML
366 showed separation between field-maintained and micropropagated plants, suggesting that both
367 types of variation could be contributing to the *in vitro* culture induced differences in MSAP profiles
368 detected. Pairwise analysis of the molecular distance (PhiST) between field maintained and
369 micropropagated plants, showed higher distances for all three genotypes when MSL where used.
370 Moreover, while differences in MSL frequencies between the propagation methods were
371 statistically significant, they were not when calculated using NML. Taken collectively, this
372 indicates that changes in DNA methylation, rather than genetic, accounted for most, if not all, of
373 the variability observed.
374

375 We then compared the band patterns of samples restricted with *Hpa*II and *Msp*I in order to assess
376 the directionally of DNA methylation change induced by *in vitro* culture (i.e. Hypermethylation

vs hypomethylation). Micropropagated plants presented higher levels of fully methylated sites and of internal cytosine methylation and lower levels of unmethylated and hemimethylated sites. This suggests that *in vitro* culture induces a global increase in DNA methylation. Previous studies have also reported higher levels of DNA methylation in tissue culture regenerants, e.g. hypermethylation on banana *in vitro* propagated clones relative to conventional ones [42], higher ratios of fully methylated CCGG sites in grapevine somaclones [43], and increased global levels DNA methylation of meristem cultures of *Malus xiaojinensis* [44].

Micropropagation induced somaclonal variation in sweet potato is genotype dependent

As discussed above, PCoA and PCA of MSAP profiles revealed that *in vitro* induced molecular variability shifted micropropagated samples from all three genotypes into the same Eigen space. This indicates that a significant portion of the *in vitro* culture-induced epialleles are shared by all plants independently of their genotype. However, our study also revealed that while Bohye and Otoo plants showed the most extensive epigenetic and genetic variability respectively, micropropagated plants from genotype Ogyefo, consistently showed to be the least affected by micropropagation, i.e. they presented 1. the lowest percentage of total variability explained by micropropagation; 2. the lowest levels of total somaclonal variation (PhiPT) and the lowest levels of both genetic and epigenetic variability (PhiST). Previous studies have shown that factors affecting the level and type of somaclonal variability include: micropropagated species, the ortet's genetic background and GC content, culture type and duration, plant hormones used, tissue used as explant material, among others [45, 46]. Here, all genotypes were exposed to the same in vitro

399 culture conditions for the same period of time, which indicates that the extent of molecular
400 variability inflicted by sweet potato micropropagation is genotype dependent.

401

402 Conclusions

403 Here we show that micropropagation reduces the nutritional value of sweet potato tubers and that
404 micropropagated plants are both genetically and epigenetically dissimilar from field maintained
405 plants. The higher levels of variability in the nutritional composition and of molecular diversity
406 observed within micropropagated plants makes tempting the speculation that there is a direct
407 relation between both. Regardless, the anonymous nature of the MSAP markers, used here to
408 characterize somaclonal variation at a molecular level, does not allow us to asseverate that the
409 detected DNA methylation polymorphisms are the drivers of the observed loss in nutritional value.
410 Still, our results provide a useful start point from which to assemble a more comprehensive picture
411 of the functional role of *in vitro* culture induced DNA methylation changes affecting the nutritional
412 value of biofortified crops. More importantly, since future sweet potato biofortification plans
413 includes the use of *in vitro* culture to generate disease free propagules, our findings highlight the
414 importance of including an assessment of the impact of micropropagation on nutritional values,
415 with a special focus on beta-carotene content, of any novel biofortified sweet potato cultivar prior
416 to their commercial release, to avoid the catastrophic costs to the industry previously seen with
417 other *in vitro* propagated crops [47].

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References

1. Baafi E, Blay ET, Ofori K, Gracen VE, Manu-Aduening J Carey EE (2016) Breeding superior orange-fleshed sweetpotato cultivars for west africa. Journal of Crop Improvement 30: 293-310.

2. Clark CA, Davis JA, Abad JA, Cuellar WJ, Fuentes S, Kreuze JF, et al. (2012) Sweetpotato viruses: 15 years of progress on understanding and managing complex diseases. Plant Disease 96: 168-185.

3. Quain MD (2001) Propagation of root and tuber crops by tissue culture in ghana. West africa seed and planting material. IITA.

4. Krishna H, Alizadeh M, Singh D, Singh U, Chauhan N, Eftekhari M, et al. (2016) Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech 6: 1-18.

5. Adu-Gyamfi R, Wetten A Marcelino Rodríguez López C (2016) Effect of cryopreservation and post-cryopreservation somatic embryogenesis on the epigenetic fidelity of cocoa (*theobroma cacao* l.). PLoS ONE 11: e0158857.

6. Kitimu SR, Taylor J, March TJ, Tairo F, Wilkinson MJ Rodriguez Lopez CM (2015) Meristem micropropagation of cassava (*manihot esculenta*) evokes genome-wide changes in DNA methylation. Frontiers in Plant Science 6.

7. Rodríguez López CM, Wetten AC Wilkinson MJ (2010) Progressive erosion of genetic and epigenetic variation in callus- derived cocoa (*theobroma cacao*) plants. New Phytologist 186: 856-868.

8. López CMR, Bravo HS, Wetten AC Wilkinson MJ (2010) Detection of somaclonal variation during cocoa somatic embryogenesis characterised using cleaved amplified polymorphic sequence and the new freeware artbio. Molecular breeding 25: 501-516.

9. Rodríguez López CM Wilkinson MJ (2015) Epi-fingerprinting and epi-interventions for improved crop production and food quality. Frontiers in Plant Science 6: 397.

10. Miguel C Marum L (2011) An epigenetic view of plant cells cultured in vitro: Somaclonal variation and beyond. Journal of Experimental Botany 62: 3713-3725.

11. Munshi A, Ahuja YR Bahadur B (2015) Epigenetic mechanisms in plants: An overview. In: B. Bahadur, et al., editors. Plant biology and biotechnology: Volume ii: Plant genomics and biotechnology. New Delhi: Springer India. pp. 265-278.

12. Elhamamsy AR (2016) DNA methylation dynamics in plants and mammals: Overview of regulation and dysregulation. Cell Biochemistry and Function 34: 289-298.

13. Xie H, Konate M, Sai N, Tesfamicael KG, Cavagnaro T, Gilliham M, et al. (2017) Global DNA methylation patterns can play a role in defining terroir in grapevine (*vitis vinifera* cv. Shiraz). Frontiers in Plant Science 8.

14. Konate M, Wilkinson M, Mayne B, Pederson S, Scott E, Berger B, et al. (2018) Salt stress induces non-cg methylation in coding regions of barley seedlings (*hordeum vulgare*). Epigenomes 2: 12.

15. Gimenez MD, Yañez-Santos AM, Paz RC, Quiroga MP, Marfil CF, Conci VC, et al. (2016) Assessment of genetic and epigenetic changes in virus-free garlic (*allium sativum* l.) plants obtained by meristem culture followed by in vitro propagation. Plant cell reports 35: 129-141.

16. Scherer RF, de Freitas Fraga HP, Klabunde GF, da Silva DA Guerra MP (2015) Global DNA methylation levels during the development of nodule cluster cultures and assessment of genetic fidelity of in vitro-regenerated pineapple plants (*ananas comosus* var. Comosus). Journal of Plant Growth Regulation 34: 677-683.

17. Osabe K, Clement JD, Bedon F, Pettolino FA, Ziolkowski L, Llewellyn DJ, et al. (2014) Genetic and DNA methylation changes in cotton (*gossypium*) genotypes and tissues. PLoS one 9: e86049.

18. Machczyńska J, Orłowska R, Mańkowski DR, Zimny J Bednarek PT (2014) DNA methylation changes in triticale due to *in vitro* culture plant regeneration and consecutive reproduction. Plant Cell Tiss Organ Cult 119: 289-299.

19. Gurmu F, Hussein S Laing M (2014) The potential of orange-fleshed sweet potato to prevent vitamin a deficiency in africa. Int J Vitam Nutr Res 84: 65-78.

20. UNICEF (2016) Unicef data: Monitoring the situation of children and women. United Nations International Children Educational Fund.

21. Sommer A (2014) Preventing blindness and saving lives: The centenary of vitamin a. JAMA Ophthalmology 132: 115-117.

22. Islam SN, Nusrat T, Begum P Ahsan M (2016) Carotenoids and b-carotene in orange fleshed sweet potato: A possible solution to vitamin a deficiency. Food Chemistry 199: 628-631.

23. Magwaza LS, Messo Naidoo SI, Laurie SM, Laing MD Shimelis H (2016) Development of nirs models for rapid quantification of protein content in sweetpotato [*ipomoea batatas* (l.) lam.]. LWT - Food Science and Technology 72: 63-70.

500 24. Pérez-Figueroa A (2013) Msap: A tool for the statistical analysis of methylation-sensitive
501 amplified polymorphism data. Molecular Ecology Resources 13: 522-527.
502 25. Agyarko K, Dapaah HK, Buah S Frimpong KA (2014) Sweet potato (*ipomoea batatas*)
503 yield parameters, soil chemical properties and cost benefit ratios following incorporation
504 of poultry manure and inorganic npk fertilizers in low nutrient ghanaian soils. International
505 Journal of Plant & Soil Science 3: 129-138.
506 26. Quain MD, Appiah-Kubi D, Adu-Gyamfi MO, Aboagye AA, Osei-Diko G, Amakwaah
507 AV, et al. (2016) In vitro production of clean planting material: Setting the time lines.
508 Research in root and tuber crops value chain development: the hope for food security in
509 the ECOWAS sub-region-WAAPP-Ghana National Centre Of Specialisation On Root And
510 Tuber Crops-Regional Conference. CSIR-Crops Research Institute: WAAPP-Ghana. pp.
511 72.
512 27. Huamán Z, International Potato C, Asian Vegetable R, Development C International Board
513 for Plant Genetic R (1991) Descriptores de la batata, descriptors for sweet potato,
514 descripteurs pour la patate douce. Rome, Italy: International Board for Plant Genetic
515 Resources. 52 p.
516 28. Amankwaah VA (2012) Phenotypic and molecular characterization of released and elite
517 sweetpotato varieties in ghana compared with virus-tested putative ramets. Department of
518 Agriculture. Kwame Nkrumah University of Science and Technology. pp. 94.
519 29. Zum Felde T, Burgos G, Espinoza J, Eyzaguirre R, Porras E Grüneberg W (2009)
520 Screening for β-carotene, iron, zinc, starch, individual sugars and protein in sweetpotato
521 germplasm by near-infrared reflectance spectroscopy (nirs). 15th Triennial Symposium of
522 the International Society for Tropical Root Crops, Lima, Peru.

30. Kim S-H Hamada T (2005) Rapid and reliable method of extracting DNA and rna from sweetpotato, *ipomoea batatas* (l). Lam. Biotechnol Lett 27: 1841-1845.

31. López CMR, Morán P, Lago F, Espiñeira M, Beckmann M Consuegra S (2012) Detection and quantification of tissue of origin in salmon and veal products using methylation sensitive aflps. Food chemistry 131: 1493-1498.

32. Peakall R Smouse PE (2012) Genalex 6.5: Genetic analysis in excel. Population genetic software for teaching and research—an update. Bioinformatics 28: 2537-2539.

33. Ruffoni B Savona M (2013) Physiological and biochemical analysis of growth abnormalities associated with plant tissue culture. Horticulture, Environment, and Biotechnology 54: 191-205.

34. Baránek M, Křižan B, Ondrušíková E Pidra M (2010) DNA-methylation changes in grapevine somaclones following *in vitro* culture and thermotherapy. Plant Cell Tiss Organ Cult 101: 11-22.

35. Peredo EL, Revilla MA Arroyo-Garcia R (2006) Assessment of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organic calli. J Plant Physiol 163.

36. Smulders MJ, Rus-Kartekaas CL Vosman B (1995) Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants. TAG Theoretical and applied genetics Theoretische und angewandte Genetik 91.

37. Machczyńska J, Zimny J Bednarek PT (2015) Tissue culture-induced genetic and epigenetic variation in triticale (× *triticosecale* spp. Wittmack ex a. Camus 1927) regenerants. Plant Mol Biol 89: 279-292.

545 38. Li X, Yu X, Wang N, Feng Q, Dong Z, Liu L, et al. (2007) Genetic and epigenetic
546 instabilities induced by tissue culture in wild barley (*hordeum brevisubulatum* (trin.) link).
547 Plant Cell, Tissue and Organ Culture 90: 153-168.
548 39. Smulders M De Klerk G (2011) Epigenetics in plant tissue culture. Plant growth regulation
549 63: 137-146.
550 40. Us-Camas R, Rivera-Solís G, Duarte-Aké F De-la-Peña C (2014) In vitro culture: An
551 epigenetic challenge for plants. Plant Cell Tiss Organ Cult 118: 187-201.
552 41. Vidalis A, Živković D, Wardenaar R, Roquis D, Tellier A Johannes F (2016) Methylome
553 evolution in plants. Genome Biology 17: 264.
554 42. Peraza-Echeverria S, Herrera-Valencia VA Kay A-J (2001) Detection of DNA methylation
555 changes in micropropagated banana plants using methylation-sensitive amplification
556 polymorphism (msap). Plant Science 161: 359-367.
557 43. Schellenbaum P, Mohler V, Wenzel G Walter B (2008) Variation in DNA methylation
558 patterns of grapevine somaclones (*vitis vinifera* l.). BMC Plant Biology 8: 1-10.
559 44. Huang H, Han SS, Wang Y, Zhang XZ Han ZH (2012) Variations in leaf morphology and
560 DNA methylation following in vitro culture of *malus xiaojinensis*. Plant Cell Tiss Organ
561 Cult 111: 153-161.
562 45. Fang J-Y, Wetten A, Adu-Gyamfi R, Wilkinson M Rodriguez-Lopez C (2009) Use of
563 secondary somatic embryos promotes genetic fidelity in cryopreservation of cocoa
564 (*theobroma cacao* l.). Agricultural and Food Science 18: 152-159.
565 46. Springer NM, Lisch D Li Q (2016) Creating order from chaos: Epigenome dynamics in
566 plants with complex genomes. The Plant Cell 28: 314-325.

567 47. Matthes M, Singh R, Cheah S-C Karp A (2001) Variation in oil palm (*elaeis guineensis*
568 jacq.) tissue culture-derived regenerants revealed by aflps with methylation-sensitive
569 enzymes. Theoret Appl Genetics 102: 971-979.

570

571

572 **Supporting Information**

573 **Fig S1. Experimental layout.** Micropropagated (shown in orange border) and field-maintained
574 plants (black bordered) planted in a common garden in a Randomized Complete Block Design
575 with three replicates. Each block consisted of three plots: Bo (Bohye), Og (Ogyefo), and Ot (Otoo).
576 Two Ridges (4.5 m long), spaced at 1 m, were made on each plot and fourteen vine cuttings of
577 about 30 cm were planted on each ridge, with an interval of 30 cm between plants. The blocks
578 were bordered with guard rows to reduce the effects of biotic and abiotic factors on the edge rows.

579 **Fig S2. PCoA results of the 12 primer combinations used for MSAP pilot studies.**

580 **Fig S3. Analysis of molecular somaclonal variation induced by micropropagation of sweet**
581 **potato. A)** PCoA generated using GenAlex v6.5 from MSAP profiles from micropropagated
582 (empty symbols) and field-maintained (full symbols) plants from genotypes of Bohye (green),
583 Ogyefo (red), and Otoo (blue) (n = 24). MSAP profiles were amplified from genomic DNA
584 restricted using *Hpa*II (circles) or MspI (squares) and amplified using primer combinations E and
585 I (Results were calculated using loci from both primer combinations together). **B)** PCA generated
586 by msapR 3.3.1 using MSAP profiles as above. Label on centroids indicate genotype (i.e. Bohye
587 (Bo), Ogyefo (Og), and Otoo (Ot)) and type of propagule (i.e. Field maintained (FM) and
588 micropropagated (VF)).

589 **Fig S4. Analysis of epigenetic and genetic variability induced by micropropagation in three**
590 **sweet potato genotypes**. PCA generated by *msap*R 3.3.1 from MSAP profiles from
591 micropropagated (blue symbols) and field-maintained (red symbols) plants from genotypes of
592 Bohye (a and d), Ogyefo (b and e), and Otoo (c and f) (n=24). MSAP profiles were amplified from
593 genomic DNA restricted using *Hpa*II and *Msp*I (profiles combined for analysis) and amplified
594 using primer combinations E (a-c) and I (d-f). Epigenetic variability was calculated using

595 Methylation Sensitive Loci (a1-f1) and genetic variability using Non-Methylated Loci (a2-f2).
596 Plants were grown on a Randomized Complete Block Design with three replicated blocks and 24
597 plants/block/genotype/propagation system.

598 **Table S1. Identity, origin, year of release, and preferred ecology of the three sweet potato**
599 **genotypes (Bohye, Ogyefo, and Otoo) used in the study.**

600 block/genotype/propagation system.

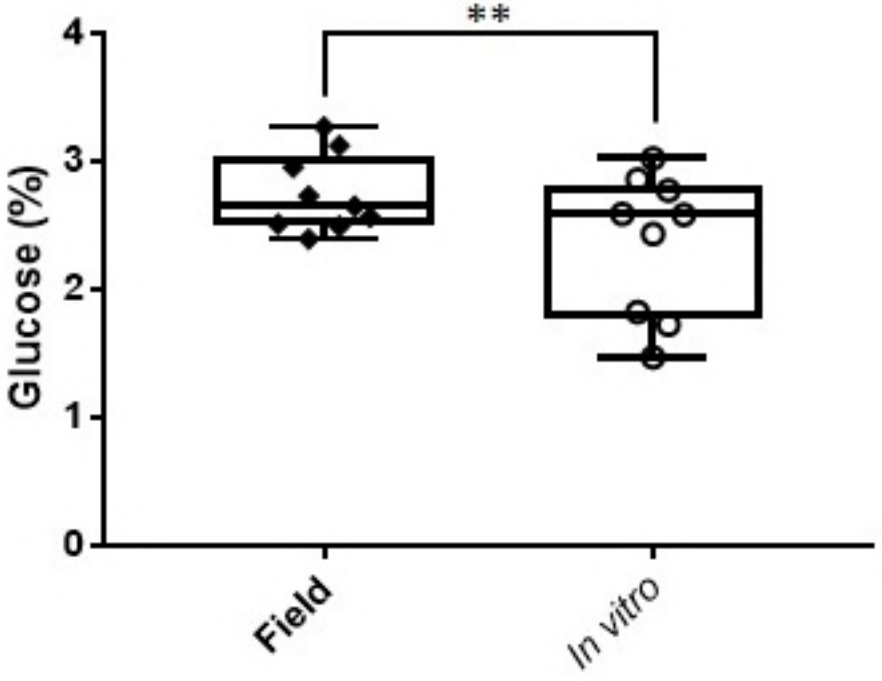
601 **Table S2** Scale of reference (1-9) and definition of scores for virus incidence, foliar and root
602 morphological descriptors.

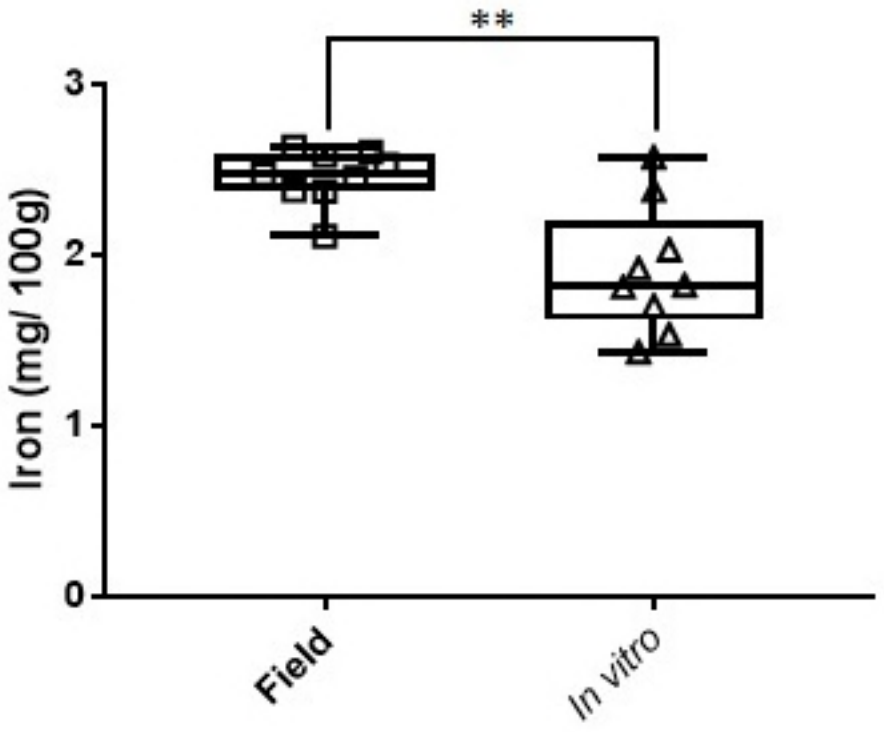
603 **Table S3.** Oligonucleotides used during Methylation Sensitive Amplified Polymorphisms protocol
604 with their sequences and function. Primer selective bases are highlighted in bold.

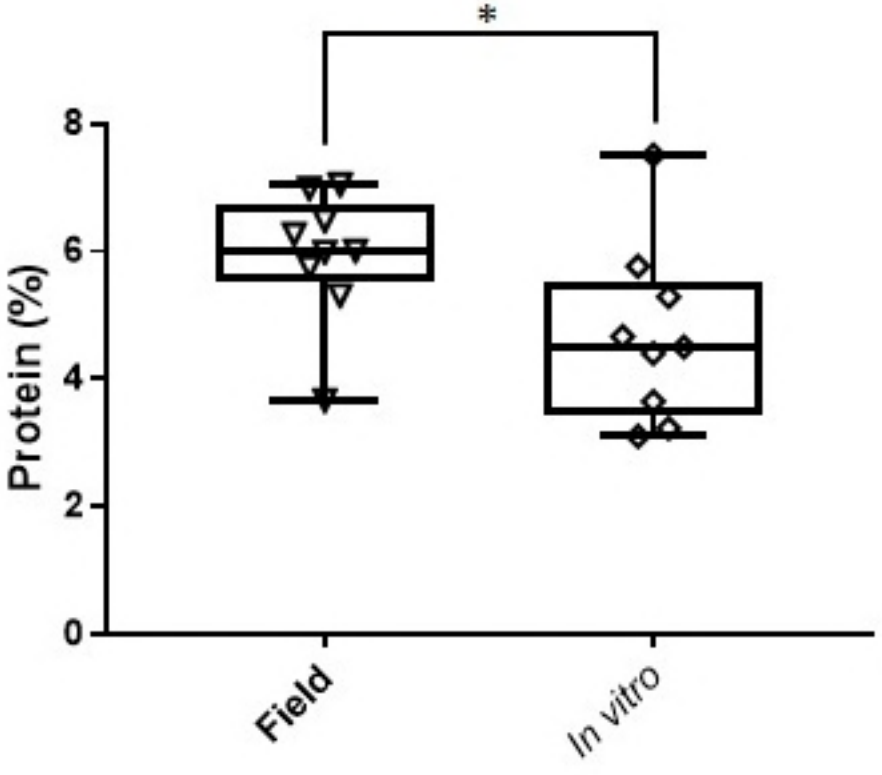
605 **Table S4.** Results of **s**elective primer combinations for MSAP pilot study. Primers selected are
606 indicated with an asterisk. The number of alleles (# of loci), percentage reproducibility of alleles
607 (% Rep), and number of differential alleles (# diff. alleles) are displayed.

608 **Table S5.** Mean foliage and root quality phenotypic scores for micropropagated (M) and field-
609 maintained (F) populations of three sweet potato genotypes Bohye, Ogyefo, and Otoo.
610 ILC=Immature Leaf Colour, MLC=Mature Leaf Colour, ALVP=Abaxial Leaf Vein Pigmentation,
611 PVC=Predominant Vine Colour, SVC=Secondary Vine Colour, PP=Petiole Pigmentation,
612 PT=Plant Type, M=micropropagated, and F=Field-maintained plants.

613


**
Glucose (%)
4
3
2
1
0
Field
In vitro


**
Iron (mg/ 100g)
3
2
1
0
Field
In vitro


*
Protein (%)
8
6
4
2
0
Field
In vitro

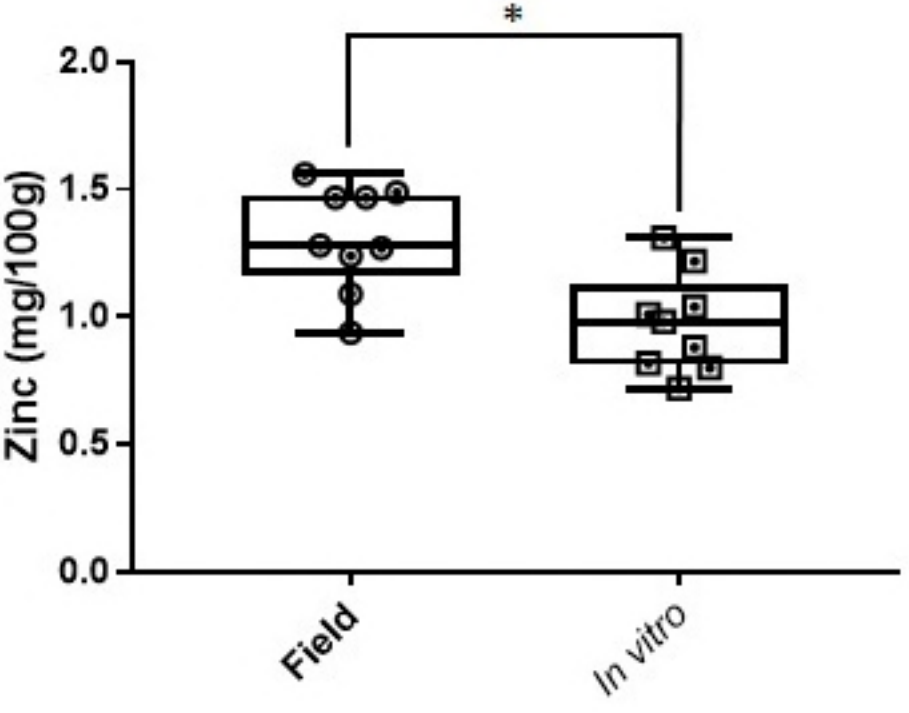

*
Zinc (mg/100g)
2.0
1.5
1.0
0.5
0.0
Field
In vitro

Figure 1

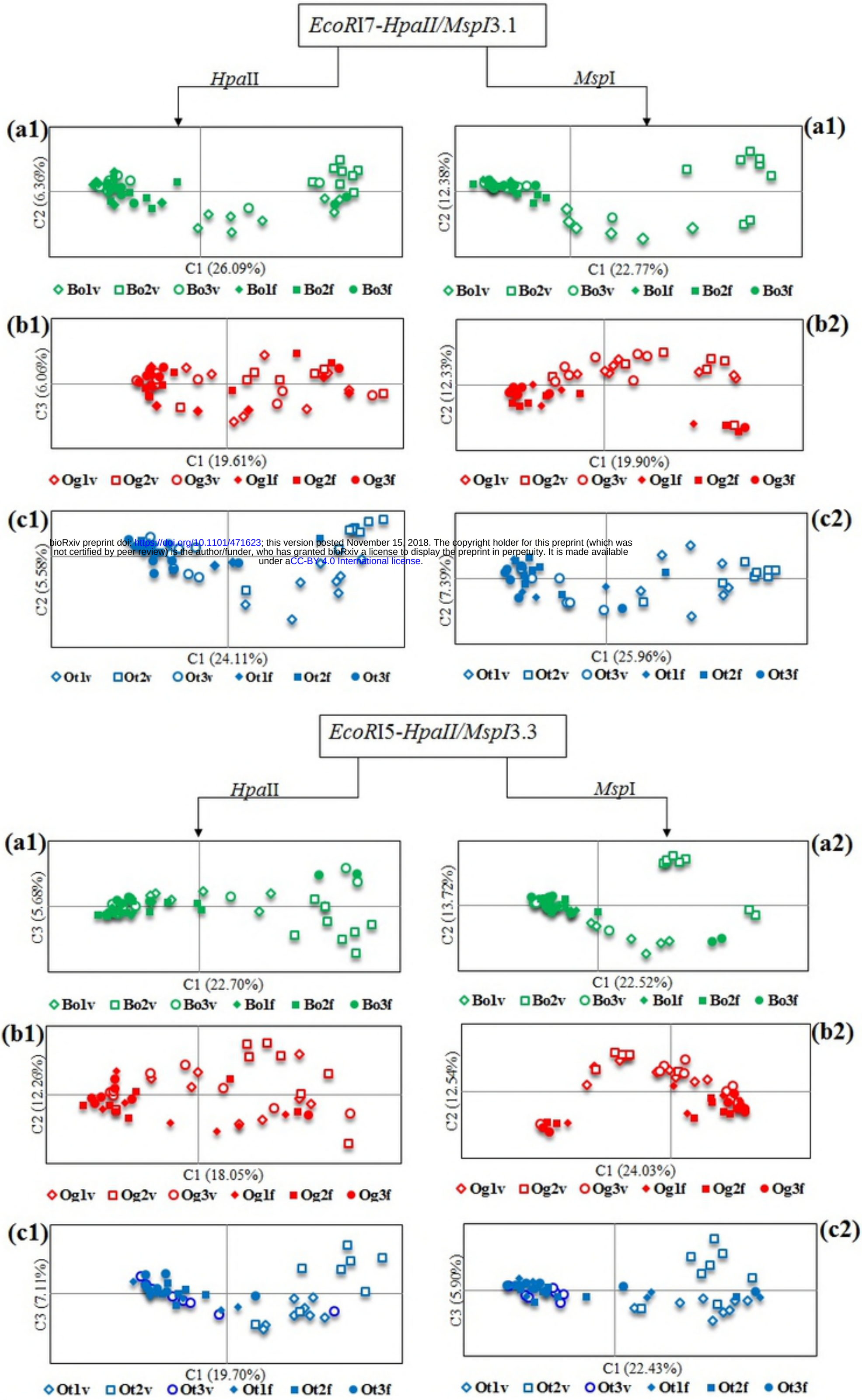


Figure 2

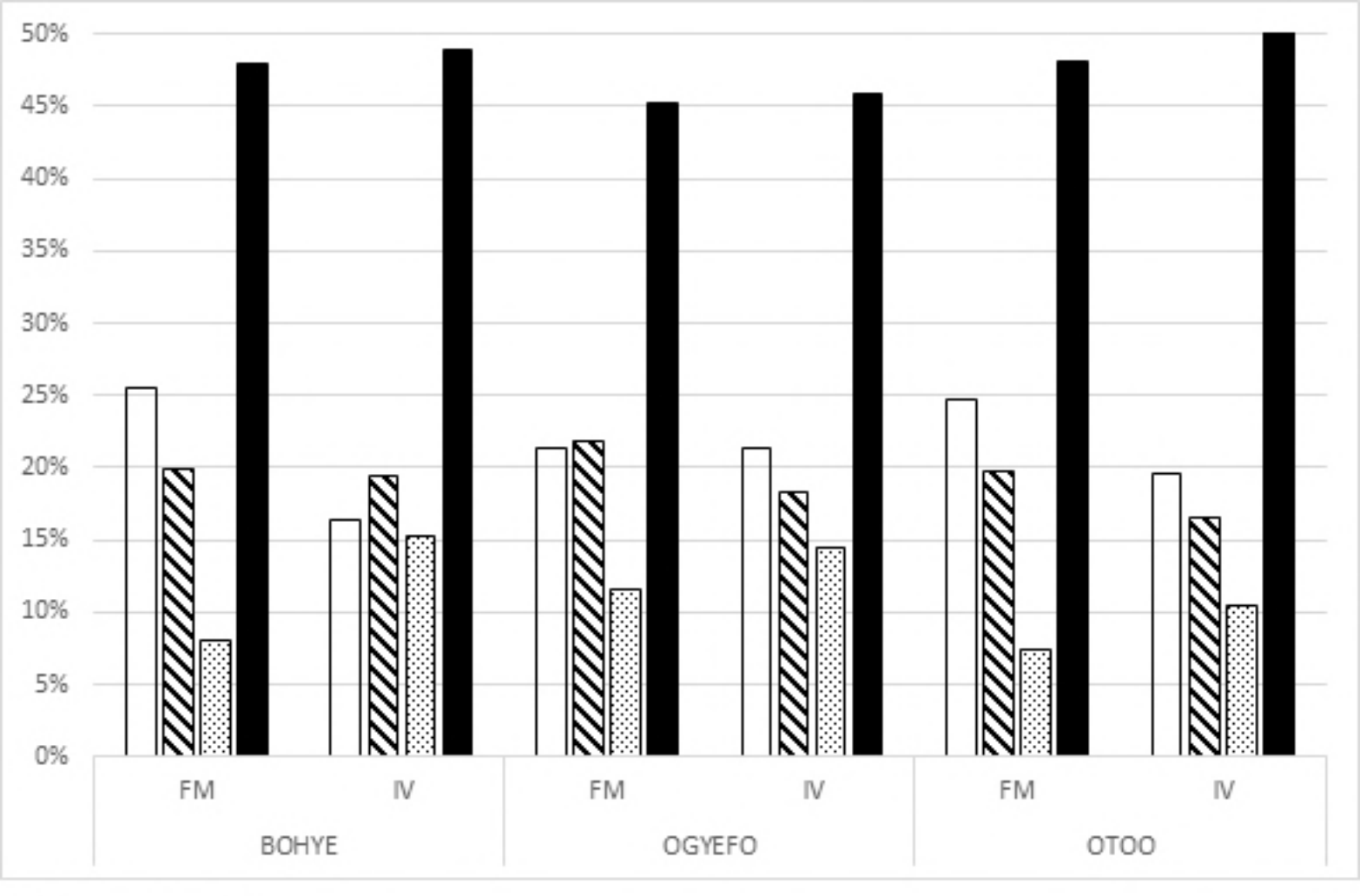


Figure 3