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Full Length Research Paper

Sampling strategies for proper quantification of carotenoid content in cassava breeding

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Rapid-cycling recurrent selection has been implemented to produce cassava varieties that deliver pro-vitamin A carotenoid to human populations that are affected by vitamin A deficiency. Rapid-cycling selection relies on quantification in just one plant per genotype. This study was conducted to assess the reliability of single plant-evaluations and the effect of age of the plant on carotenoid content in cassava roots. Variation in aliquot quantifications from the same root was negligible indicating a reliable experimental procedure. A large source of variation for carotenoids was due to differences among genotypes. Root-to-root variation from the same plant in some cases was surprisingly high and accounted for an average of 25% of the total variation. Plant-to-plant variation was not as high and accounted for 20% of the total variance. Carotenoid content was shown to vary depending on the age of the plant. Single-plant evaluations for carotenoid content in cassava, which is a requirement for rapid-cycling recurrent selection is acceptable considering that it reduces in half the time required for evaluation and selection. However, it is suggested that 2 to 3 roots per plant are combined together in a sample to represent each genotype at a standard plant age.

Key words: Plant age, environmental influence, rapid cycling, genetic gains, β-carotene, repeatability.

INTRODUCTION

Vitamin A (VA), along with iron and iodine, is among the most important micronutrients whose deficiency is a matter of public health concern (Underwood, 2000). It is estimated that 190 million preschool-age children have low serum retinol (<0.70 µmol L^{-1}), the sub-clinical symptom of VA deficiency (WHO, 2009). Improving the VA status of children reduces mortality rates by 23 to 30% (ACC/SCN, 2000; Beaton et al., 1993; West, 2003). There is growing evidence that VA has a positive synergistic effect with iron and zinc bio-availability (Graham and Rosser, 2000). VA is the generic descriptor for compounds with the qualitative biological activity of retinol. VA exists as preformed retinoids (retinol, retinal and retinoic acid) which are stored in animal tissue and pro-VA carotenoid, which are synthesized and stored in many green, yellow and/or orange plant tissues. Carotenoids from vegetables contribute two-thirds of dietary VA, worldwide, and more than 80% in the developing world (Combs, 1998).

Three main strategies have been traditionally used to prevent VA deficiency: Dietary diversification, food fortification and/or supplementation. These strategies are relatively cost-effective, but have failed to completely eradicate the problem for a diversity of reasons (West, 2003). Recently, different programs (HarvestPlus, AgroSalud) involving a global alliance of research institutions initiated the development of a fourth strategy (biofortification) to develop micronutrient-dense staple crops (Hirschi, 2008; Pfeiffer and McClafferty, 2007a, b; Welch and Graham, 2005). Among these initiatives is the development of biofortified cassava varieties with high pro-VA contents in the roots. Biofortification can be achieved through conventional breeding techniques that

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Abbreviations: TCC, total carotenoids content; **TBC,** all-trans-β-carotene; **FW,** fresh weight; **DW,** dry weight; DMC, dry matter content; **UV,** ultra violet; **CV,** coefficient of variation; **MAP,** months after planting.

take advantage of the genetic variability for micronutrients in different crops (Latham, 2003; Welch, 2002; Chávez et al., 2005). It represents a sustainable strategy that aims at solving the root of the micronutrient problem: A deficient diet. However, the higher micronutrient content of biofortified crops needs to be retained after processing the food (industrially and/or in the home) ultimately leading to greater absorption and use by the body. Several studies are gradually contributing to a better understanding of carotenoid retention in different biofortified crops (Li et al., 2007; Chávez et al., 2008; van Jaarsveld et al., 2006). Recent studies are also contributing to our understanding of the efficiency of carotenoid conversion present in cassava (*Manihot esculenta* Crantz) roots and other crops into VA (Thakkar et al., 2007, 2009; Failla et al., 2008; Liu, 2009; van Jaarsveld et al., 2005).

Cassava is an important food source for more than 70 million people in developing countries that obtain more than 500 kcal/day from cassava roots (Cock, 1985; Kawano, 1998). However, current cassava varieties produce roots with low levels of protein, fat, minerals and micronutrients such as pro-VA carotenoid.

A normal breeding cycle in cassava lasts for about 6 to 8 years since the botanical seed of segregating progenies are germinated until the best genotypes among these segregating progenies can be definitively identified (Ceballos et al., 2007). This long breeding cycle is required because most relevant variables are quantitatively inherited and are strongly affected by the environment, showing large genotype-by-environment interaction effects. However, heritability of carotenoid content in cassava roots is relatively high (Morillo, 2009). Previous research suggested a good uniformity of carotenoid along and across cassava roots, among roots from the same plant and between roots of different plants from the same genotype (Chávez et al., 2008). Studies on genotype-by-environment interaction for carotenoid content in cassava demonstrate that the interaction is statistically significant but does not result in drastic changes of the relative ranking of the different genotypes (Ssemakula and Dixon, 2007).

A rapid-cycling recurrent selection scheme was therefore implemented by the cassava project at the International Center for Tropical Agriculture (CIAT) (Morante et al., 2009). In this scheme, thousands of segregating progenies were evaluated every year and those with high-carotenoid content were crossed to initiate a new cycle of selection. The botanical seed produced was then germinated and the resulting seedlings transplanted to the field for evaluation. Plants were sampled at the standard age of 11 to 12 months after planting. One root was taken from the standing plant and was immediately analyzed for its carotenoids content. Selection was first made visually for color intensity (selecting for intense yellow coloration of the parenchyma and discarding white or cream genotypes), then for total carotenoids content using the spectrophotometer data and then the carotenoids extracts from the best samples were analyzed with HPLC for quantification of the different carotenoid pigments. Genotypes with high carotene were thus identified while the mother plant remained growing in the field until the finalization of the evaluations. Stems from the selected genotypes were then harvested and incorporated into the crossing blocks. Therefore, within 2 years, progenies from these elite genotypes (which would be a new cycle of recurrent selection) could be harvested and screened. Distinctive features of this scheme were that (typically) one root from the single plant representing each genotype is used to quantify carotenoid content, and that roots were taken from a mother plant that remained in the field until the entire trial had been screened.

The ultimate purpose of this study was to develop reliable sampling strategies for proper quantification of carotenoid in segregating progenies and thus maximize genetic gains when a rapid-cycling recurrent selection approach is used. The specific objectives were to determine the variation in carotenoid content in roots from the same cassava plant and in different plants from the same genotype, including quantification at three different ages of the plant.

MATERIALS AND METHODS

Germplasm

Two different studies are described in this article as shown below. In both experiments, germplasm from the CIAT cassava-breeding project was selected based on the availability of planting material and to cover a wide range of variation for carotenoid contents. The materials used were all experimental clones developed by the project. Planting was done using vegetative cuttings about 20 cm long and with no less than five nodes. Cultural practices, planting times, fertilization and irrigation followed the standard approach recommended for cassava by CIAT (Calle, 2002; Cadavid, 2002).

Harvest and root handling

Roots were harvested at different ages of the plant, depending on the experiment as described below. Plants were pooled from the ground manually and roots were cut from the crown of the plant and selected based on their health, good appearance and commercial size (ranging from 400 to 800 g, 20 to 30 cm long and 5 to 10 cm in diameter). Handling of the roots was done carefully trying to prevent physical damage. Roots were not stored. Harvesting was done at sunrise and carotenoid extraction in the morning hours. Quantification of total carotenoid content was done before noon and HPLC quantifications, if made, were carried out in the afternoon. Root samples and extracts were protected from the light as much as possible. How was it done? Answer: In the carotene extraction section, we provided some information, which has now been expanded to answer this question.

Roots were cut longitudinally in half and then the two halves again longitudinally into quarters. Each quarter would include, therefore, tissue from the periphery, mid-parenchyma and core of the root, as well as proximal, central and distal sections (Chávez et al., 2008). Two opposite quarters of the roots were pooled together for carotenoid quantification and the remaining quarters were used for dry matter quantification (or other root quality traits such as cyanogenic potential). This approach neutralized differences along

and across the root for carotenoid content (as demonstrated by Chávez et al., 2008). The two quarters of each root were then properly ground and mixed for a uniform and representative sample.

Dry matter content

A sample from these roots was taken for the quantification of dry matter content (DMC). To estimate it, 20 to 30 g of chopped and grated fresh roots were dried in an oven at 60°C for 24 h. Dry matter was expressed as the percentage of dry weight relative to fresh weight.

Carotenoid extraction

Carotenoids were extracted following the method suggested in the literature (Rodriguez-Amaya, 2001; Rodriguez-Amaya and Kimura 2004), except that separation of the solid and liquid phases was carried out by centrifugation and not by filtration (Chávez et al., 2005). Carotenoids are sensitive to ultraviolet (UV) light, air and any pro-oxidants or associated compounds, and high temperature. Thus, steps were taken to avoid any adverse changes in this pigment due to such effects protecting them from UV light and avoiding excessively high temperatures. Special care was taken to avoid direct exposure to sunlight and the lights in the laboratory were protected with UV filters. Samples were covered with paper or aluminum foil when not under processing. Approximately 5 g of fresh root tissue were homogenized for 1 min with 10 ml acetone: Petroleum ether (1:1) using a Polytron homogenizer (IKA T18, Staufen, Germany), followed by centrifugation (Eppendorf 5804R, Hamburg, Germany), at 3000 rpm, for 10 min, at 4ºC. The liquid phase was collected and extraction of the residue, followed by centrifugation, was repeated until it turned colourless (usually 3 times). The extracts were then combined with 10 ml of 0.1 M NaCl solution and the petroleum ether phase containing the carotenoids separated from the lower aqueous-acetone phase.

Carotenoid quantification

With the extracts obtained, total carotenoid content (TCC) was determined by visible absorption spectrophotometry (Cecil CE2021, Cambridge, UK), at an absorbance of 450 nm and using the absorption coefficient of β-carotene in petroleum ether (2592) (Rodriguez-Amaya, 2001; Rodriguez-Amaya et al., 2004). All-trans-β-carotene (TBC) quantification was done by HPLC. From the petroleum ether solution used for spectrophotometric quantification of total carotenoid, aliquots (15 ml) were taken, partially dried by rota-evaporation (Laborota 4000, Schwabach, Germany) and completely dried with nitrogen. Immediately before injection, the dry extract was dissolved in 1 ml of Methanol:Methy tert-butyl Ether (1:1) HPLC-grade and filtered through a 0.22 µm PTFE syringe filter. Separation and quantification of carotenoid were achieved using an YMC Carotenoid S-5 C30 reversed-phase column (4.6 × 150 mm: Particle size, 5 µm), with a YMC Carotenoid S-5 guard column (4.0 × 23 mm) in a HPLC system (Agilent Technologies 1200 series, Waldbronn, Germany), with a cooling auto-sampler unit at 4°C to avoid evaporation of the injection solvent and degradation of carotenoids by heat and DAD detector with wavelength set at 450 nm. Peaks were identified by comparing retention time and spectral characteristics against a pure standard and available literature. Quantity was determined by integration of peak area against a standard curve prepared with known concentrations of all-trans-β-carotene. TCC and TBC were estimated on a fresh (TCC-FW and TBC-FW) and dry weight (TCC-DW and TBC-DW) basis.

Before making any determination, the method was previously validated according the requirements of Thompson et al. (2002) and EURACHEM (2000). The method used has linearity (correlation coefficient) of 0.99925 at 2, 6, 10, 14, 18, 25, 30 ppm levels, and three replicates. The relative standard deviation in repeatability conditions is 2.1% and the reproducibility estimated in terms of intermediate is lower than 13.6% with a percent of recovery of 94.5% and also CIAT participated in an inter-laboratory study involving several laboratories world wide. Results from this study confirmed adequate precision of quantified carotenoids from sweet potato and cassava (CIAT, 2007).

Study 1: Sampling variation among plants and roots from the same genotype

A total of 35 cloned cassava genotypes were used in this study. Roots were harvested when plants were 11 months old and their TCC quantified. These genotypes were selected because they offered a wide range of carotenoid contents (based on evaluations made at the seedling stage the previous season). Roots from each of two plants representing each genotype were harvested in November 2008 and three of them were selected based on their health and commercial size. Two measurements per root (aliquots) were made, for a total of 12 measurements in most genotypes (only 18 samples could be analyzed per day and there were a total of 420 potential samples). Roots from the same plant and plants from the same genotypes were harvested and analyzed in the same day.

Statistical analysis to partition components of variation was conducted using the variance components estimation procedure of SAS analytical software (Cary, NC, USA). Coefficients of variation (CV) were calculated using the 6 data points (obtained after averaging the two aliquots per sample) from each genotype. CV was estimated based on the relationship between the standard deviation of the 6 samples analyzed divided by the average of the genotype and expressed as percentage (Steel and Torrie, 1960). A compromise was made in the design of this study based on the number of samples that can be processed per day, the number of plants per genotype and the number of roots per plant. A relatively large number of genotypes were desirable to screen for this variability and therefore the number of plants per genotype and the ideal number of roots per plant was set to be three.

Study 2: Variation of carotenoid content at different ages of the plant

A second experiment was conducted with 54 cassava clones harvested at 8, 10 and 12 months after planting (MAP). These genotypes were different from those used in the first study and include many different crosses among different high-carotenoid progenitors. For each genotype, 2 plants and 2 to 3 roots from each of them were harvested and combined for a single sample per genotype. Planting of this experiment was done in August, 2008 and harvest took place in March, May and August, 2009.

Two main sources of variability were considered (clone and harvesting date). The analysis of variance included the interaction between these two main sources of variation, which were considered fixed effects. Analysis of variance, coefficient of variations and least significant differences (LSD) values were estimated using the Statistix 8 software (Tallahassee, FL, USA).

RESULTS

Study 1: Sampling variation among plants and roots from the same genotype

From the 35 clones initially planted for this experiment,

Table 1. Variability between roots from the same plant and between plants from the same genotype for dry matter content (DMC), total carotenoid contents on a fresh weight basis (TCC-FW,) and on a dry weight basis (TCC-DW).

Parameter	Quantified variable			Pl. 1 vs. Pl. 2[2]	Coefficient of variation		
	Mean	Max	Min		Mean	Max	Min
DMC (%)	34.13	42.32	26.07	2.65	7.81	22.70	2.11
TCC-FW (µg g^{-1})	7.99	12.95	2.87	1.29	15.75	59.54	3.99
TCC-DW (µg g^{-1})	23.36	33.86	10.27	3.61	15.05	52.39	1.97

Sampling was based on two plants per genotype and three roots per plant for most of the 35 genotypes evaluated. Two quantifications per root were made for carotenoids content[1].[1]Max; maximum; Min: minimum; Pl: Plant, [2]Average difference between the results of roots from the two plants used to represent eac

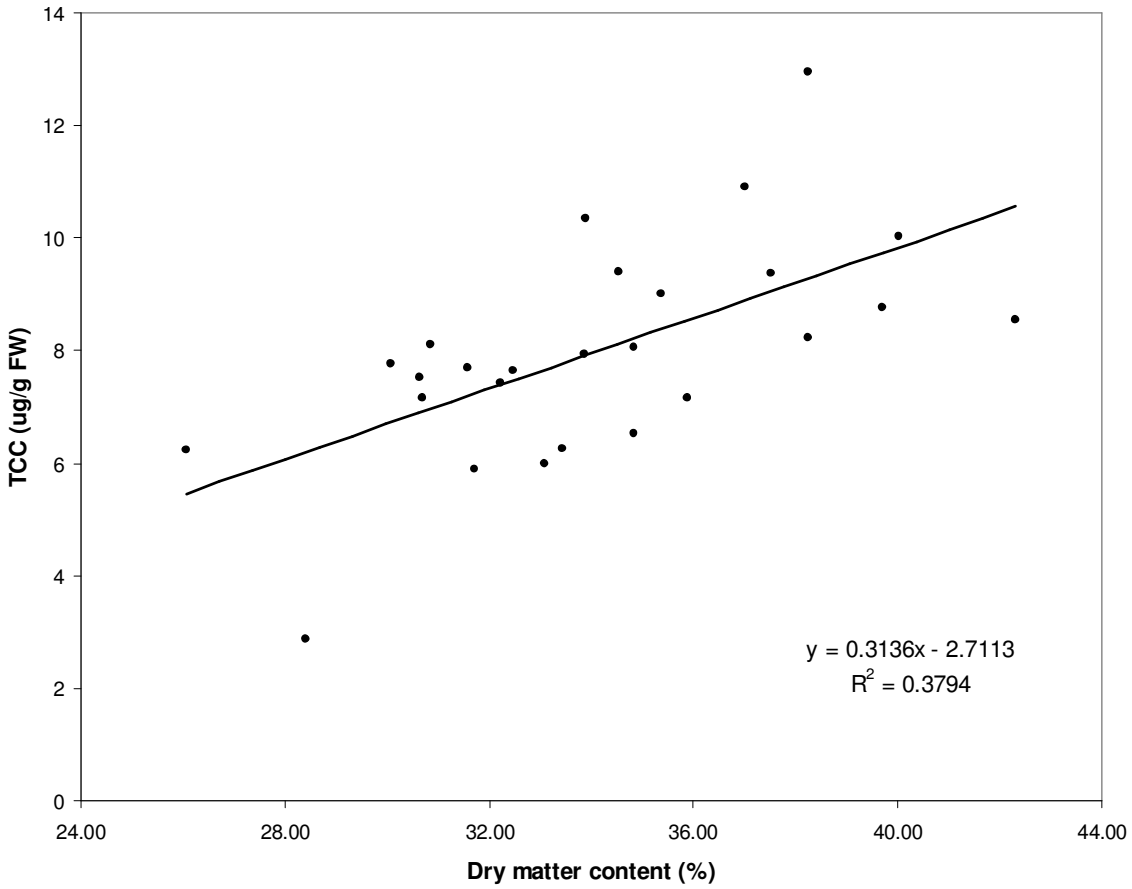

Figure 1. Relationship between dry matter content (%) and TCC-FW (µg g^{-1} fresh root) in the 26 genotypes analyzed (data points are averages across roots within a plant and the two plants per genotype).

only 26 allowed the harvest of at least 2 commercial-size roots from each of 2 plants. 17 genotypes allowed the harvest of 3 roots from the 2 plants for a total of 6 different root samples. In the case of three genotypes, 3 roots were harvested in one plant, but only 2 in the second. From the remaining six genotypes, only 2 roots from each of the 2 plants could be harvested and analyzed. The total number of roots analyzed, therefore was 141 and since 2 aliquots per root were used, the total number of analyses was 242. Average difference between the 2 aliquots for TCC was 0.47 µg g^{-1} fresh roots.

Table 1 presents the average, range of variation and coefficient of variation for DMC, TCC-FW and TCC-DW for each of the 26 genotypes analyzed (based on sampling two plants per genotype, ideally three roots from each of these two plants, and two aliquots per root). Figure 1 illustrates the relationship between DMC and TCC-FW (based on the averages for each genotype). There is a clear positive association between the two

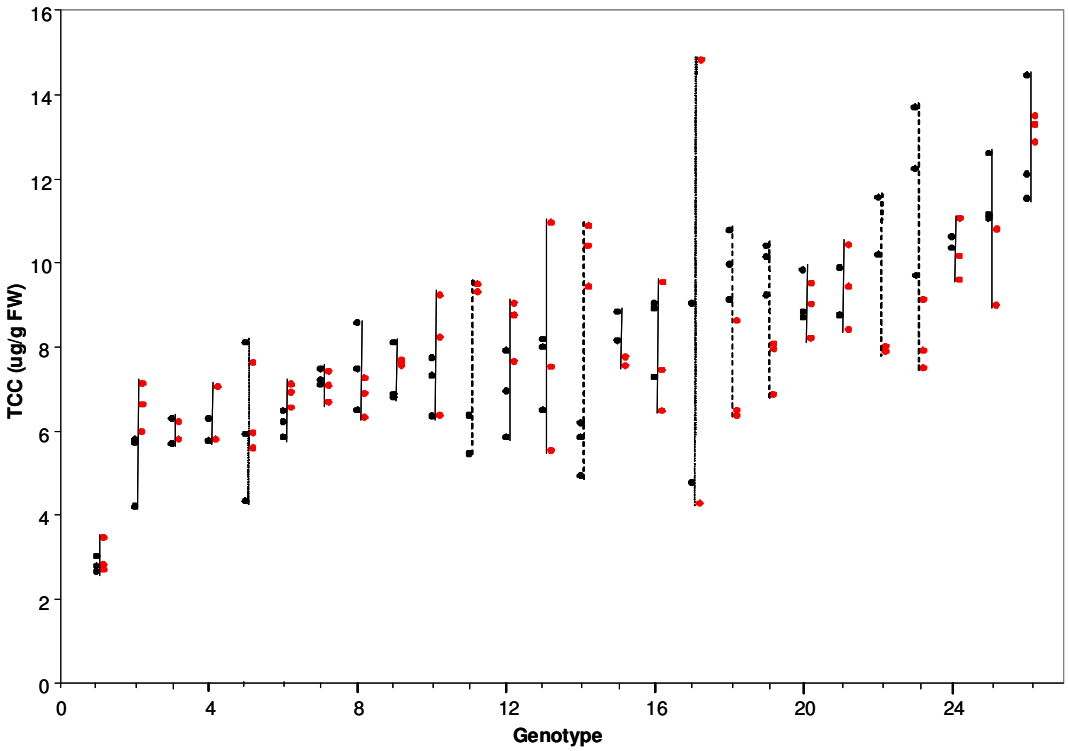

Figure 2. Total carotenoid content in roots from 26 genotypes. Up to three roots from two plants (red or black dots) were harvested. Dots linked with a line represent roots from the same genotype. Dotted lines identify clones with large variation among the roots sampled from the two plants representing each genotype.

Table 2. Variance components for dry matter content (DMC), total carotenoid content on a fresh weight basis (TCC-FW,) and on a dry weight basis (TCC-DW).

Source of variation	Variable		
	DMC (%)	TCC-FW (µg g^{-1})	TCC-DW (µg g^{-1})
Clone	11.76 (53.4)	3.17 (54.2)	16.91 (42.8)
Plant-to-plant (clone)	4.32 (19.7)	1.16 (19.8)	9.31 (23.4)
Root-to-root (clone*plant)	5.34 (24.4)	1.34 (22.9)	11.47 (29.1)
Error (aliquots)	0.51 (2.3)	0.18 (3.1)	1.80 (4.6)

Sampling was based on two plants per genotype and three roots per plant for most of the 35 gentoypes evaluated. Two quantifications per root were made. [1]Within parenthesis the percentage of the total variation accounted for each of the components.

variables. A wide range of variation for the average TCC could be observed among the 26 clones (from a minimum of 2.87 to a maximum of 12.95 µg g^{-1} fresh root). When plant-to-plant, and root-to-root variation (within the plant) were analyzed, differences were often negligible but in few cases, there was a significant variation (Figure 2).

Table 2 provides information on variance components for each of the variables analyzed. As expected, the most important component was the genetic differences among the 26 clones evaluated (more than 50% of the total variation was accounted for by this source). Differences between the two aliquots taken from each root were very small and accounted for less than 5% of the total variation in the experiment. The remaining sources of variation were due to plant-to-plant variation for each clone and for root-to-root variation within a plant. The root-to-root variation was higher than the plant-to-plant variation (accounting for an average of about 25 and 20%, respectively, of the total variation measured for each of the three variables analyzed).

The average CVs for each genotype is also provided in Table 1. These CVs relate the standard deviation between the six roots analyzed in most genotypes and their respective average. For TCC-FW, one genotype

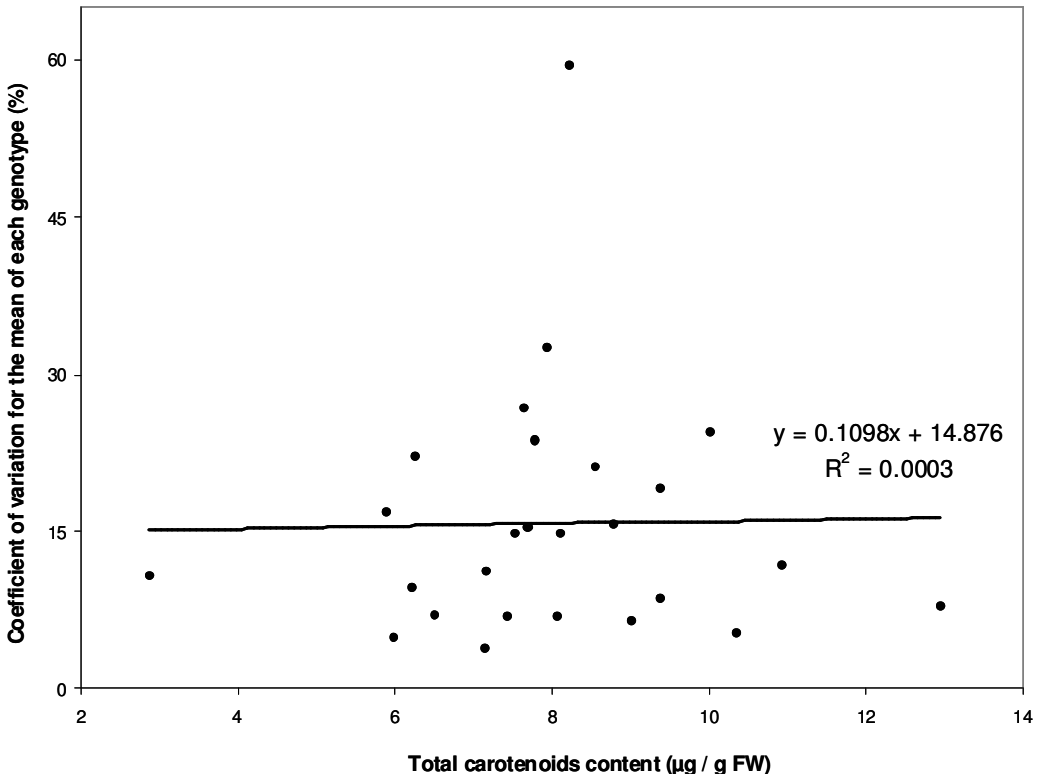

Figure 3. Relationship between average total carotenoid content (µg g^{-1} FW) in roots from 26 genotypes and the respective coefficient of variation (%). The high CV point corresponds to genotype 17 in Figure 2.

(genotype 17) showed a very high CV (60%). This is apparent from the plot provided in Figure 2. The second highest CV for TCC-FW (genotype 14) was considerably lower (33%). Four genotypes had a CV between 20 and 30% (Genotypes 5, 13, 18 and 23). The remaining 20 genotypes had CV values below 20% for TCC-FW, which is much more acceptable (What are your interpretations based on the low CVS?).

There were seven genotypes where the difference in the average TCC-FW for the two plants was relatively large. The two plants from Genotype 14 (Figure 2) showed very contrasting averages (a difference as large as 4.59 µg g^{-1} FW). Consistent, but smaller, differences between the two plants of genotypes 23, 18 and 19 could also be observed (differences were 3.71, 2.79 and 2.30 µg g^{-1} FW, respectively). The plant-to-plant variation observed in these genotypes contributed to their high CV values. In all these cases, three roots could be harvested from each plant, and root-to-root variation within each plant was relatively low. Therefore the difference between the averages of the two plants representing each genotype was considered to be real. Only two roots from each of the two plants representing genotypes 11 and 22 (Figure 2) could be harvested. The average TCC for the two plants differed by 3.49 and 2.96 µg g^{-1} FW, respectively.

Results from genotype 17 were surprising. Root-to-root variation within one plant was very large (from 4.27 to 14.8 µg g^{-1} FW). The range of variation for the roots of the second plant was not as large. In other cases, such as in genotypes 5 and 13 relatively large root-to-root variation (> 3 µg g^{-1} FW) within one plant (but not the other) could be observed.

Experimental errors for TCC-FW using different sampling strategies were estimated. The average experimental error for TCC-FW considering data from one plant was 0.405 µg g^{-1} FW (this would be for observations based on three roots and two aliquots). The addition of data from a second plant marginally reduced the value of experimental error to 0.389 (error for values based on 12 data points: 2 plants, 3 roots per plant and two aliquots). Similar conclusions can be drawn for DMC and TCC-DW.

Most of the genotypes showing large differences in TCC-FW also showed large differences when TCC was expressed as dry weight (data not shown). Therefore, variation between plants could not be explained (and, therefore, corrected), as originally hoped, by differences in DMC.

Figure 3 illustrates the relationship between average total carotenoid content (µg g^{-1} FW) in roots from 26 genotypes and the respective coefficient of variation (%). It is clear that there is no increase in the degree of variation between samples as TCC values increase.

Table 3. Results from the analysis of variance for carotenoids and dry matter contents of 54 cassava clones harvested at 8, 10 and 12 months after planting.

Variable	Harvest (month after planting)[1]			CV	LSD	
	8	10	12	(%)	(P < 0.05)	(P < 0.01)
Dry matter content (%)	34.25	28.21	25.54	7.84	0.618	0.816
TCC (µg g^{-1} FW)	8.91	9.55	9.31	11.44	0.283	0.373
TCC (µg g^{-1} DW)	26.0	34.0	36.8	11.81	1.018	1.343
TBC (µg g^{-1} FW)	6.48	6.85	6.45	13.62	0.239	0.316
TBC (µg g^{-1} DW)	18.96	24.32	25.56	13.56	0.829	1.095
TBC (% TCC)	70.26	69.98	68.51	6.97	1.303	1.720

[1] For each of the 54 genotypes, up to six plants were planted. For each sampling time 2 to 3 roots from each of two plants were harvested and combined for a single sample. Values provided in these columns are the averages for dry matter content (DMC), total carotenoids (TCC) and total beta carotene (TBC)

Study 2: Variation of carotenoid content at different ages of the plant

Table 3 shows the average results at different ages for DMC, TCC-FW, TCC-DW, TBC-FW (all-trans-β-carotene, fresh weight basis), TBC-DW (all-trans-β-carotene, dry weight basis), and TBC% (TBC-FW, expressed as percentage of TCC-FW) to monitor if the relative proportion of different carotenoid would shift with the age of the plant.

Analysis of variance indicated highly significant differences (P < 0.01) for clones, dates of harvest and their interaction effects for all the variables measured, except for the harvesting date main effect for the TBC% variable, which was significant only at the 5% probability level.

Data presented in Table 3 showed surprising results for DMC. Roots harvested 10 and 12 MAP had an unexpected reduction in DMC. The maximum DMC level was attained 8 MAP (34.25%) and decreased successively at 10 MAP (28.21%) and 12 MAP (25.54%). These differences were significant at the 1% probability level. TCC-FW and TBC-FW increased from 8 to 10 MAP (significant difference at 1% probability level) but then decreased slightly at 12 MAP. Averages between 10 and 12 MAP were not statistically different from each other. On the other hand, these variables when expressed on a dry weight basis (TCC-DW and TBC-DW) increased consistently from 8 to 10 and to 12 MAP (Table 3). Differences between these averages were always statistically significant at the 1% probability level.

TBC% apparently decreased with the age of the plant following the same pattern as DMC. However, the differences between the averages at 8 and 10 MAP and between 10 and 12 MAP were not statistically significant. Difference of TBC% between 8 and 12 MAP was statistically significant at the 1% probability level (Table 3). Coefficients of variation ranged from 6.97% for TBC% and 13.62% for TBC-FW, indicating adequate quality of the data.

DISCUSSION

The low variation between the two aliquots per root in the first study (Table 2) is a clear indication of the precision quantifying TCC in cassava roots. Therefore, the experimental procedure to extract and quantify carotenoid is clearly adequate. The rest of the discussion will concentrate on sampling variation. In spite of the differences detected in some genotypes thoroughly described in the results section, TCC-FW is generally stable within a clone and does not require a large number of roots or plants per genotype. Cases where root-to-root variation within a plant and/or plant-to-plant variation within a genotype were high were the exception rather than the rule. The kind of variation found in some of the genotypes evaluated in this study had not been detected in an earlier work (Chávez et al., 2008). One plausible explanation for the contrast of the results between these two studies is that Chávez et al. (2008) evaluated just one genotype whose levels of TCC were relatively low (3.90 µg g^{-1} FW). In the present study, however, several genotypes were evaluated and many of them had considerably higher TCC values than the genotype evaluated by Chávez et al. (2008).

It is difficult to explain the kind of variation quantified in root samples from certain genotypes. For example, the large variation observed for genotype 17 had been expected because, upon harvest, personnel had visually noticed the contrasting degree of pigmentation in the parenchyma in this particular genotype. For some reason, therefore, some roots of a given plant failed to accumulate considerable amount of carotenoid, whereas other roots from the same plant had done the opposite. The only feasible explanation which has become our working hypothesis is that in a few genotypes, root growth was not uniform: Some roots initiated the bulking process of starch (and carotenoid) accumulation relatively early and other roots late in the life of the plant. Roots that have initiated their growth earlier would have more time to accumulate carotenoid and at harvest time

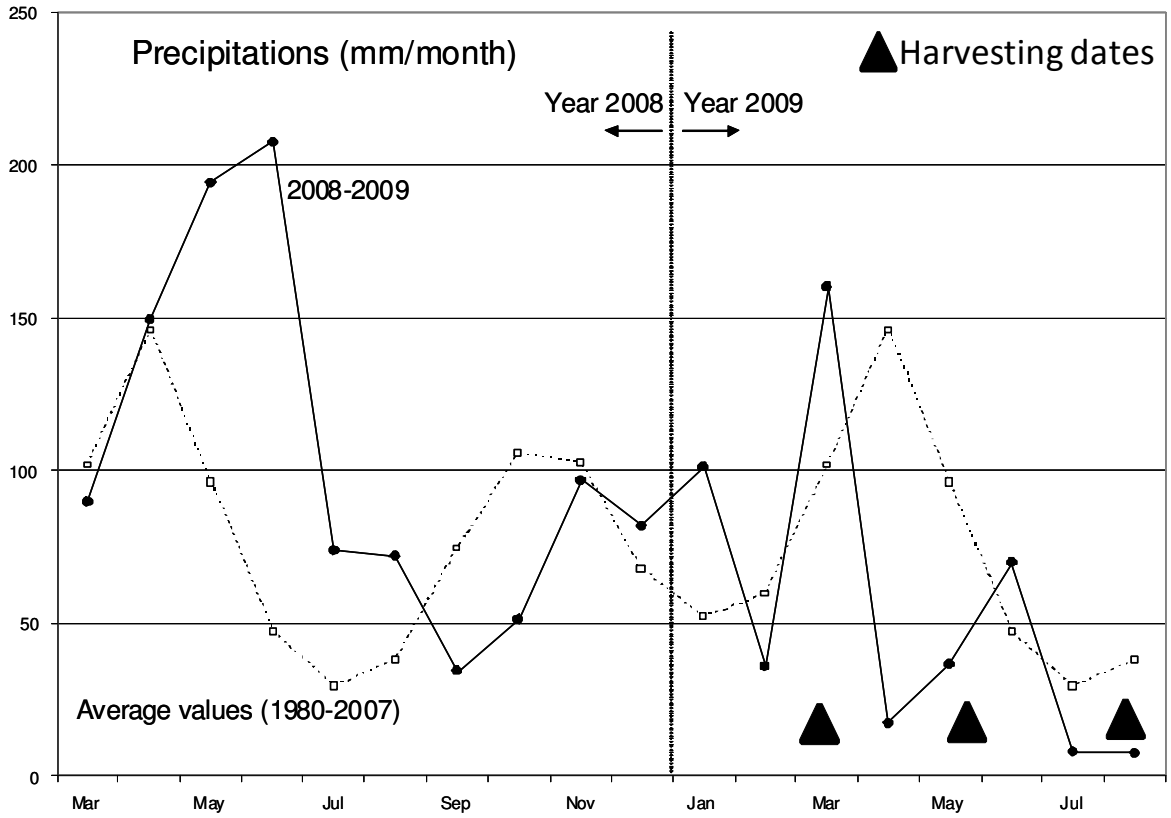

Figure 4. Data on rainfall in the period March 2008 to August 2009 compared with historic averages at CIAT experimental station in Palmira, Colombia (from 1980 through 2007).

would have higher concentrations of this pigment.

Information provided in Figures 1 and 3 is worth emphasizing. It has been occasionally mentioned in different scientific forums that there is a negative correlation between DMC and TCC-FW. Figure 1, however, clearly demonstrates that this is not the case. Moreover, there is a clearly positive association between the two variables. One additional concern that needed to be addressed was the possibility that as breeding work improved the levels of TCC in cassava roots, the reliability of the quantifications would be lower by increased degree of variation among roots and plants from the same genotype. The information in Figure 3, however, demonstrates that the magnitude of coefficients of variation was not dependent on the TCC-FW values.

Results presented in Table 3 from the second study to analyze the effect of age of the plant on carotenoid content were surprising. In general, harvest of cassava plants takes place at 10 to 12 MAP, when DMC reaches a maximum and farmers can obtain a maximum return for their product. In this study, however, DMC declined successively from 8 to 10 and to 12 MAP.

Figure 4 provides a comparison of historic data (1980-2007) of monthly rainfall and current data from March 2008 through August 2009 (dates when the second experiment was initiated and completed, respectively). Data were obtained from a weather station at the CIAT Experimental Station where the experiments took place. There was an obvious and drastic change in the climatic conditions throughout the duration of the experiment. Many different environmental factors were analyzed (such as evapotranspiration, heliophany, air temperature, etc.) but the most drastic change is related to the shift of rainfall patterns. Particularly relevant were the unusual conditions from March to August, 2009. Historic data suggest a bimodal distribution of rains with two peaks, one in April and the second in October-November. After the first harvest at 8 MAP, when DMC showed an acceptable level of 34.25% rains rather than reaching the expected maximum in April (historic average of 146 mm) were almost negligible (18 mm in April 2009). In June 2009, actual precipitation (70 mm) was almost double the expected for this time of the year (47 mm). These unusual climatic patterns can explain the unexpected behavior of DMC at the different harvesting dates. During dry spells, cassava plants drop their leaves and assume a dormant stage until the arrival of the rains. When this happens, the plant hydrolyzes the starch (and other compounds) accumulated in the roots to reinitiate growth. This process therefore, leads to a drastic reduction in DMC (van Oirschot et al., 2000).

The second study described in this article aimed at

confirming and measuring what cassava breeders knew empirically: Carotenoid content (expressed on a fresh weight basis) increases with the age of the plant. But this expectation was drastically affected by the unusual variation in DMC. The reduction in DMC "diluted" the carotenoid accumulated in the roots. It had been expected to have a "normal" pattern for the evolution of DMC: Increasing from 8 to 10 MAP and then, perhaps, measure a slight decline at 12 MAP (after the typical drop of rainfall in July, which during the current study was much more drastic). But this unusual DMC evolution affected TCC and TBC data. The average TCC-FW at 10 MAP should have been higher than the 9.55 µg g^{-1} quantified. Similarly, at 12 MAP, TCC-FW should have been higher than the 9.31 µg g^{-1} figure obtained. When total carotenoid and all-trans-β-carotene were expressed as dry weight basis, however, values meet the expected pattern of a gradual and consistent increase with the age of the plant (Table 3).

The interaction between the age of the plant and genotype sources of variation in the second experiment was statistically significant. However, the discussion focused on the variation related to age of the plant because the main interest of this study was to demonstrate that this factor influences TCC-FW and, therefore, has to be taken into account. The significance of the interaction implies additional sources of variation that are difficult for the researchers to predict a 'priori'. The relevance of the second study is to highlight, as expected, the impact of age of the plant in carotenoid content in cassava roots. Moreover, the second experiment also provided evidence for the importance of dry matter content as an indicator variable to assess the quality of TCC-FW data in cassava roots. Although results are still preliminary (regarding our initial hypothesis that TCC-FW and TBC-FW increase with age of the plant from 8 to 10 to 12 MAP) we can definitively conclude that age of the plant affects carotenoid content in cassava roots and therefore, the former needs to be taken into account for data to be comparable.

It has to be recognized that for the two studies herein reported, there was a limitation in the number of samples that could be analyzed. This is a major bottleneck in the few breeding projects aiming at developing cassava genotypes with high-carotenoids in their roots. In ordinary breeding work as well as in these two studies, only a limited number of samples can be analyzed each day. In our studies, we have tried to analyze all the roots from a given genotype the same day to maximize the uniformity of the conditions for these evaluations and avoid any potential source of variability arising from assessments made in different days. The main objective of this study, however, was to demonstrate the need of improving the sampling protocols and to highlight the indirect influence of the environment through variation in dry matter contents. This has already been accomplished in spite of the limited number of samples used.

Conclusions

It is not possible to predict beforehand which plant will show differences in carotenoid concentration among its roots as was observed in a few cases in the first study as illustrated in Figure 2. The relevance of this study, therefore, is to suggest a change in sampling procedures. Chávez et al. (2008) suggested that sampling one root from one plant per genotype would suffice for the quantification of TCC-FW in cassava roots. Results from the current study suggest that this recommendation has to be corrected. This study demonstrated for the first time that, in some cases (impossible to predict which ones), there was larger than acceptable variation; it is advisable to take more than one root per plant since about 25% of the variation arises from the root-to-root variation within a plant as presented in Table 2. If there is more than one plant per genotype available, it may be advisable to sample 2 to 3 roots per plant. Samples from different roots could then be combined in a single sample per genotype. However, this additional effort in the sampling procedure will only marginally reduce the experimental errors. It should be pointed out however, that the fields at CIAT's Experimental Station where the studies were conducted are fairly uniform. If evaluations are conducted in more variable conditions, the influence of plant-to-plant variation may be higher.

In the case of the ongoing rapid-cycling recurrent selection project to increase carotenoid content in cassava, evaluation and selection takes place at the seedling plant stage (plants that come from germinated botanical seeds which have not been cloned). There is no possibility to have more than one plant per genotype. To have a more accurate assessment of the TCC value for each genotype would require extending the duration of each cycle of evaluation for an additional year (so quantification could be made on cloned plants). The improved degree of precision in a few genotypes (that is, reducing the experimental error from 0.405 to 0.389 µg g^{-1}), however, does not compensate duplicating the length of the evaluation phase (and therefore reducing the rate of genetic gains time wise). It is therefore sensible to recommend that the current strategy of evaluating and selecting at the seedling stage is maintained but with the modification that 2 to 3 commercial-size roots should be used in a combined sample to represent each genotype.

The fact that the storage organ of cassava is a root and not a tuber has important implications (Alves, 2002). The root does not reach a "physiological maturity" where quality parameters would tend to be more stable and predictable. Root quality characteristics of cassava vary with the age of the plant and the environmental conditions (Sriroth et al., 1999). For most cases, relevant root quality traits are predictable and stable if age of the plant is taken into consideration. Environmental conditions and cultural practices, however, may have an important impact through changes in DMC (van Oirschot

et al., 2000). It is recommended, therefore, to analyze TCC at a standard age of the plant and to use DMC as an indicator variable of the growing conditions of the plant and the reliability of root quality parameters such as TCC-FW.

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