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# Differing Sucrose Requirements for *In-vitro* Conservation of Cassava Genotypes

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author RA designed the study and wrote the protocol. Author HYS conducted the experiment and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.*

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## ABSTRACT

Field conservation of vegetative propagated crops poses a major problem to curators of germplasm, especially in developing countries. An alternative method to ensure security of germplasm is the use of tissue culture techniques in media formulated for slow growth. However, tissues of plant species may require different nutrients for optimum growth. The objectives of this study were to a) assess the effects of sucrose on the performance of different cassava genotypes and b) recommend sucrose levels for in-vitro conservation of the genotypes. Four sucrose levels (0, 10, 20, and 30 g $l^{-1}$) and apical meristems of five cassava genotypes (Bankye Hemaah, Bankye Botan, Tek Bankye, Doku Duade, and Essam Bankye) cultured in-vitro was studied at the Kwame Nkrumah University of Science and Technology's plant biotechnology laboratory. Growth media were prepared using hormone-free Murashige and Skoog (MS) basal media formulation. Inoculated cultures were exposed to 16 hours of light and 8 hours of darkness with light illuminance of 3500 lux and also maintained at 24±2°C temperature and relative humidity of 70%. All genotypes

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showed a direct regeneration without callus formation. Generally, sucrose enhanced the growth performance of plantlets; however, the genotypes responded differently to sucrose in leaf formation, plant height, and rooting ability with time. For long term conservation, growth medium must sustain the health of plantlets with infrequent need for sub-culturing. As such, sucrose levels of 10 g $l^{-1}$ for Essam Bankye, 20 g $l^{-1}$ for Doku Duade and Tek Bankye, and 30 g $l^{-1}$ for Bankye Hemaa and Bankye Botan were the recommended rates for in-vitro conservation.

*Keywords: Cassava; germplasm conservation; tissue culture; slow growth.*

## 1. INTRODUCTION

Field conservation of vegetative propagated crops poses a major problem to curators of germplasm, especially in developing countries. This is because of losses from pests and diseases, drought stress, wild fires, and thefts associated with it. Alternative method to ensure security of germplasm is the use of tissue culture techniques in media formulated for maintenance under slow growth. However, tissues of plant species have different nutrient requirements for satisfactory growth [1]. As such, one medium cannot be optimum for different plant genotypes [2-4].

For conservation of germplasm, media that will ensure slow growth, while sustaining health of plantlets are required. In addition, the technique has to be relatively cheaper and easier to adopt as high cost of production is a major limitation of tissue culture adoption in developing countries [5-7], especially in Ghana where this study was conducted. Among the different *in-vitro* slow growth methods available (e.g., reduction of sucrose as energy source, temperature regulation, inclusion of hormones that retard growth, and use of cryopreservation), reduction of sucrose is, arguably, one of the cheapest and easiest to adapt, suggesting the need to optimize sucrose levels for *in-vitro* conservation of different plant species.

Considerable attention has been given to germplasm conservation of different crops in Ghana. One important crop is cassava (*Manihot esculenta* Crantz), which is the second most important source of carbohydrate for meals after maize in the world [8]. Cassava and yam contribute about 46% of the agricultural Gross Domestic Product in Ghana [9]. In addition, cassava is cultivated by most farming family in Ghana and it accounts for 30% daily calorie intake [9]. Optimizing *in-vitro* low cost and easy to adapt slow growth methods for cassava will be useful for a) conservation of rare and endangered genotypes b) production of true-to-type plants and c) international exchange of germplasm.

Recommended sucrose level for *in-vitro* propagation of cassava is 30 g $l^{-1}$ [3,6]. However, it might be possible to achieve slow growth without significant effect on the health and vigor of *in-vitro* cassava plantlets by reducing the sucrose level. As such, the objectives of this study were to a) assess the effects of sucrose on the performance of different cassava genotypes and b) recommend sucrose levels for *in-vitro* conservation using hormone free Murashige and Skoog (MS) basal medium.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Materials

Stem cuttings of five cassava genotypes (Bankye Hemaah, Bankye Botan, Tek Bankye, Doku Duade, and Essam Bankye) were obtained from Kwame Nkrumah University of Science and Technology's Plantation Division of the Department of Crop & Soil Sciences, Ghana. They were cut into 4 to 6-node sections and planted in approximately 1.5-litre pails filled with loamy soil. The pails were kept in lath house to avoid a direct contact of the sprouts with rain which could serve as a source of contaminant.

### 2.2 Excision and Disinfection of Explant

A scalpel was used to excise 1-2 cm long apical shoots from the mother plants and transferred to a clean $GA_7$ vessels containing distilled water. The shoots were rinsed four times with sterile distilled water for five to ten minutes. All visible leaves were removed with a scalpel and rinsed one more time with sterile distilled water. The explants were transferred to laminar flow hood, disinfected with 70% ethanol for one minute, followed by rinsing with three changes of sterile distilled water. Disinfection was continued with 10% sodium hypochlorite containing four drops of Tween-20 (Polyoxyethylene (20) sorbitan

monolaurate) for 15 minutes, and after, rinsed with several changes of sterile distilled water. Use of Tween-20 allowed the sterilant to be in good contact with all surfaces of the tissue.

### 2.3 Culture Media Preparation

MS basal medium of 1 liter was prepared and 3.5 g phytagel was added while the media was placed on a hot magnetic stirrer which provided heat to dissolve the phytagel. Four lots were prepared and sucrose concentrations of 10, 20, and 30g were added to each medium as the treatments and no sucrose served as the control. The pH of the media was adjusted to 5.8 and dispensed into test tubes in aliquots of 10ml each while they were hot. The dispensed media in test tubes were covered and autoclaved at a temperature of 121°C and 103.4 kPa pressure for 15 minutes. The media was allowed to cool after autoclaving and then stored in a refrigerator at 4°C.

### 2.4 Isolation of Meristem Tips and Inoculation

Sterilized pairs of forceps were used to hold the sterilized meristem tips over a stereo microscope stage. A 15X magnification lens was selected and using a surgical blade, the over primordial leaves were removed one at a time with caution. The process was continued until the meristematic dome and the first pair of leaf primordial was exposed. A v-shaped cut was made approximately 0.5 mm below the tip of the dome so as to include a pair of primordial and some adjacent tissues, making a total of about 2 mm size explants. The explants were immediately removed and inoculated onto the culture media, using one explant per test tube. The entire process was carried out under the laminar flow hood to minimize contamination. Also, the surgical blade and the pair of forceps were sterilized after every explant was cultured to avoid cross contamination.

Inoculated cultures were transferred to a growth chamber and were exposed to 16 hours of light and 8 hours of darkness with light illuminance of 3500 lux for root and shoot development. The growth chamber was maintained at 24±2°C temperature and relative humidity of 70%.

### 2.5 Experimental Management and Statistical Analysis

The experiment was a two factor factorial arrangement of the five genotypes and four sucrose concentrations in a completely randomized design with ten replications. The entire experiment was repeated twice to improve reliability of the results. Data were collected for ten weeks, ensuring consistency in the day and time of day, by measuring the number of leaves, roots, and height of the plantlets biweekly.

Validity of normality, equal variance, and independence assumptions on the error terms were confirmed by assessing the residuals [10]. Analysis of variance test was performed using the PROC MIXED procedure in SAS 9.4 [11]. Genotype, sucrose level, and time were considered fixed effects, and replication and their interactions were considered random effects. Orthogonal polynomial contrast analysis was performed to determine the type of response exhibited by the cultivars over time with differing sucrose concentrations. Significant levels were determined at $P = .05$. Based on the growth and general health of plantlets, different sucrose levels were recommended for *in-vitro* conservation of the various genotypes, and the recommended rates used to sub-culture the plantlets (2-node stem cuttings).

## 3. RESULTS AND DISCUSSION

### 3.1 General Growth Performance of Genotypes

Signs of survival, showing greening of explants, occurred within five days after inoculation. All genotypes showed direct regeneration without callus formation. Sucrose enhanced growth performance of plantlets relative to the control treatment in all variables tested in this study (Table 1). Bankye Hemaa and Bankye Botan showed poor plant growth and vigor when cultured in media prepared with sucrose level below 30 g $l^{-1}$. However, the health and vigor of Doku Duade and Tek Bankye plantlets were sustained in growth media supplemented with 20 g $l^{-1}$, while exhibiting slow growth process. Generally, Essam Bankye showed the highest response in all growth parameters and still performed well in media formulated with 10 g $l^{-1}$ sucrose.

**Table 1. Sucrose effects on number of leaves, number of roots, and plant height of cassava genotypes after culturing *in-vitro* for ten months in hormone free Murashige and Skoog basal media using apical meristem**

| Sucrose levels (g $l^{-1}$) | Number of leaves | | | | | Plant height (cm) | | | | | Number of roots | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Doku Duade | Bankye Hemaa | Essam Bankye | Tek Bankye | Bankye Botan | Doku Duade | Bankye Hemaa | Essam Bankye | Tek Bankye | Bankye Botan | Doku Duade | Bankye Hemaa | Essam Bankye | Tek Bankye | Bankye Botan |
| 0* | 1.77c | 1.63c | 1.93d | 1.53c | 1.33c | 0.833d | 0.600d | 0.867c | 0.667d | 0.533d | - | - | - | - | - |
| 10 | 2.27c | 2.13c | 2.67c | 1.93c | 1.63c | 1.53c | 1.13c | 1.73b | 1.37c | 1.03c | 1.37b | 1.17b | 3.77c | 1.13c | 0.833c |
| 20 | 3.80b | 3.67b | 3.97b | 3.57b | 2.30b | 1.92b | 1.43b | 2.13b | 1.78b | 1.30b | 2.67a | 2.40a | 6.50b | 3.03b | 1.40b |
| 30 | 5.07a | 4.94a | 5.27a | 4.63a | 3.13a | 2.43a | 1.8a | 2.80a | 2.28a | 1.57a | 3.30a | 3.03a | 9.13a | 4.07a | 1.97a |
| CV (%) | 9.74 | 10.80 | 8.17 | 11.02 | 7.97 | 8.96 | 8.57 | 9.58 | 9.93 | 8.90 | 13.31 | 13.60 | 13.41 | 10.38 | 13.47 |

*Within column; means followed by same letter(s) are not significantly different using the least squares means (LSMEANS) and adjusted Tukey multiple comparison procedure (P =.05), CV=Coefficient of variation;*
**Served as control treatment in the experiment*

Sucrose is the major source of carbon and energy for plants cultured *in-vitro* [12]. In addition, concentrations of carbon source in a growth medium for in-vitro cultures plays an important role in the growth regulation and shoot elongation of plantlets [13]. Our current study, which sought to induce slow growth in cassava meristem-tip-derived plantlets for an increased conservation interval between sub-cultures, confirmed that sucrose elicited better growth. Research results indicate that enhancement of morphogenesis caused by nitrogen is only apparent when there is adequate sucrose concentration in growth medium [14], which was observed in the control treatment in our present study. However, the genotypes responded differently to varying sucrose levels.

There was little influence of sucrose on the number of shoots formed. Only few plantlets of Essam Bankye produced multiple shoots after culturing. The other genotypes did not produce multiple shoots. This was confirmed by Kozai et al. [15] who reported that sucrose is not a necessary requirement for shoot formation of *in-vitro* plantlets in conditions of high air exchanges and carbon dioxide. Nonetheless, a significant increase in the number of shoots formed was related to increased number of sub-cultures in another study [16].

### 3.2 Genotype and Sucrose Effects on Leaf Formation of Cultures

There was a significant genotype × sucrose level × time interaction effect on the number of leaves formed (*P<.0001*). All the genotypes showed cubic response with time when the medium was prepared with 20 and 30 g $l^{-1}$ sucrose (Fig. 1). With the exception of Tek Bankye that showed quartic response with time, all the genotypes exhibited a cubic response when the growth medium was supplemented with 10 g $l^{-1}$ sucrose. For the control treatment, the genotypes (Bankye Botan and Bankye Hemaa-quadratic; Doku Duade-cubic; Essam Bankye and TekBankye-quartic) showed different responses with time.

High sucrose concentration has been reported to increase the number of leaves formed by plantlets cultured *in-vitro* [17]. On the contrary, another research [18] found that high sucrose concentration inhibited leaf formation in fern, and the authors recommended 30g $l^{-1}$ sucrose as the optimum level. In enriched carbon dioxide media, functional chloroplast can produce carbohydrate by undergoing photosynthesis. This has been shown to raise sucrose amounts in media to levels that can inhibit growth and health of plantlets [19]. However, species have different functional chloroplast and subsequent ability to produce carbohydrate. Production of carbohydrate by functional chloroplast might explain why Essam Bankye plantlets were able to perform well at low sucrose levels in our current study.

### 3.3 Plant Height as Affected by Genotype and Sucrose

Genotype, sucrose level, and time showed a significant interaction effect on the height of plantlets (*P<.0001*). Bankye Hemaa showed cubic response with time at all levels of sucrose concentration (Fig. 2B). Bankye Botan, Doku Duade and Tek Bankye showed cubic responses with time at 10, 20, and 30 g $l^{-1}$ sucrose level, but increased linearly with time in media prepared without sucrose (Figs. 2A, 2D and 2E). However, Essam Bankye showed a quadratic response in media prepared without sucrose, and a cubic response when media contained sucrose (Fig. 2C). Generally, the higher the sucrose level, the longer the cultured plantlets. This corroborates the results of previous studies [20] which assessed the effects of different sucrose levels (10, 20, 30, 40 and 50 g $l^{-1}$) on *Gloriosa rothschildiana* when cultured *in-vitro* with MS basal media. The author observed longest shoots in media supplemented with the highest sucrose level.

### 3.4 Effects of Sucrose Level on Rooting Ability of Cultures

Genotype × sucrose level × time interaction effect was significant on the number of roots formed (*P<.0001*). Root initiation occurred five weeks after tissue culturing, but no root initiation was observed in media formulated with 0 g $l^{-1}$ for all the genotypes (Table 1). When media were supplemented with 10 g $l^{-1}$ sucrose; Doku Duade, Bankye Hemaa, and Tek Bankye exhibited a quadratic response with time; however, Bankye Botan, and Essam Bankye showed significance at the fourth order polynomial (Fig. 3). All genotypes showed quartic responses with time when 20 g $l^{-1}$ was used, except Bankye Botan that showed a cubic response (Fig. 3). Also, Bankye Botan, Doku Duade, and TekBankye showed cubic responses at 30 g $l^{-1}$ sucrose, whereas Bankye Hemaa and Essam Bankye exhibited quartic responses for the same sucrose level (Fig. 3).

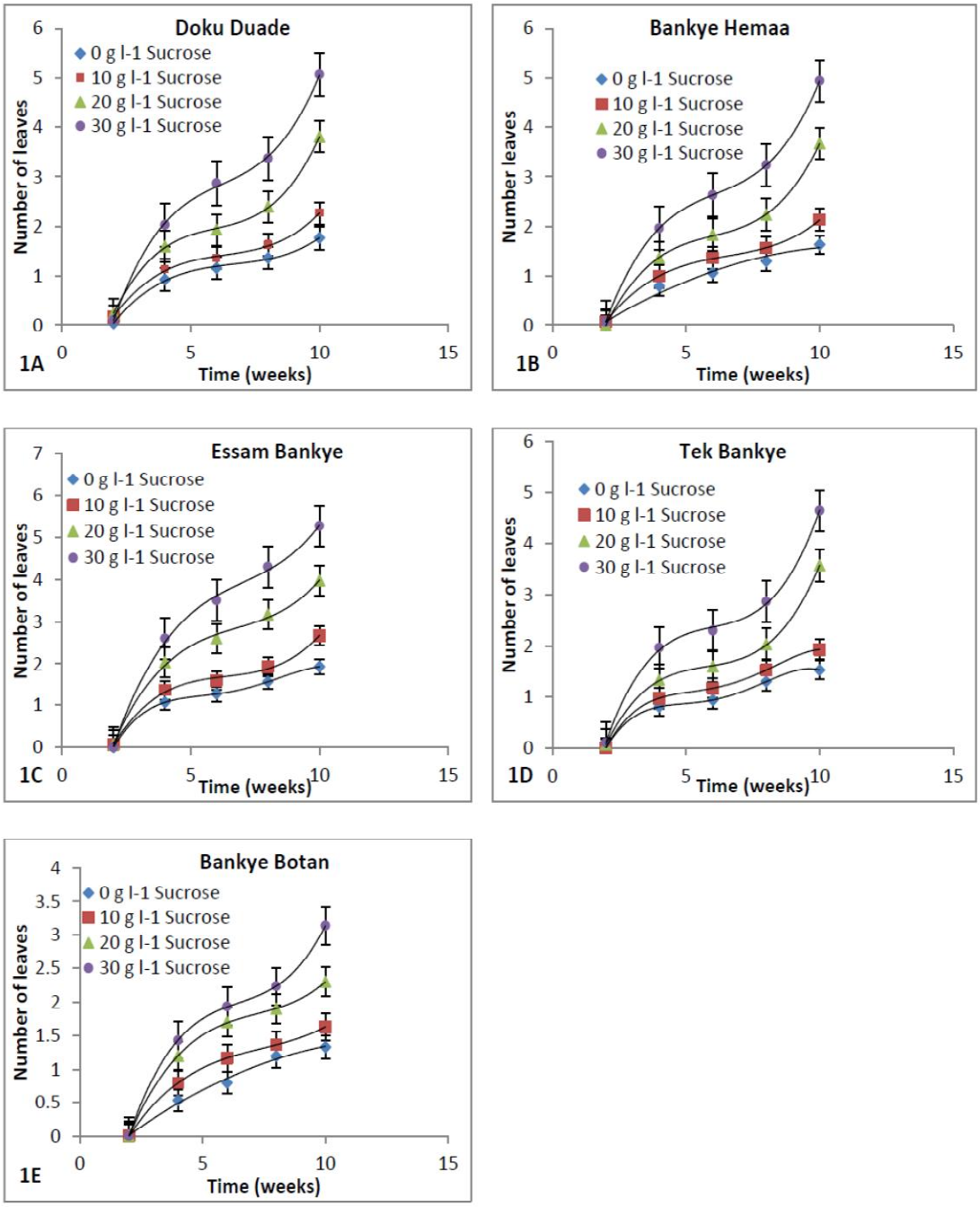


**Fig. 1. Sucrose and time effects on leaf formation of the cassava genotypes cultured *in-vitro* using apical meristem in hormone free Murashige and Skoog basal media. Response curves were determined by orthogonal polynomial contrast analysis determined at *P=.05***

Previous studies have determined that media devoid of sucrose do not produce roots [12,21], which conforms to findings from our study. This is because root initiation is a high energy formation process that requires metabolic substrate, usually carbohydrate. In addition, Stamp and Henshaw [22] reported differential response of cassava cultivars to root formation when cultured *in*-vitro, which shows genotype specificity for sucrose requirement. For long term

conservation, growth media used must sustain health of plantlets with infrequent need for sub-culturing. Based on general growth and health of the plantlets, sucrose levels of 10 g $l^{-1}$ for Essam Bankye, 20 g $l^{-1}$ for Doku Duade and Tek Bankye, and 30 g $l^{-1}$ for Bankye Hemaa and Bankye Botan were the recommended rates for *in-vitro* conservation.

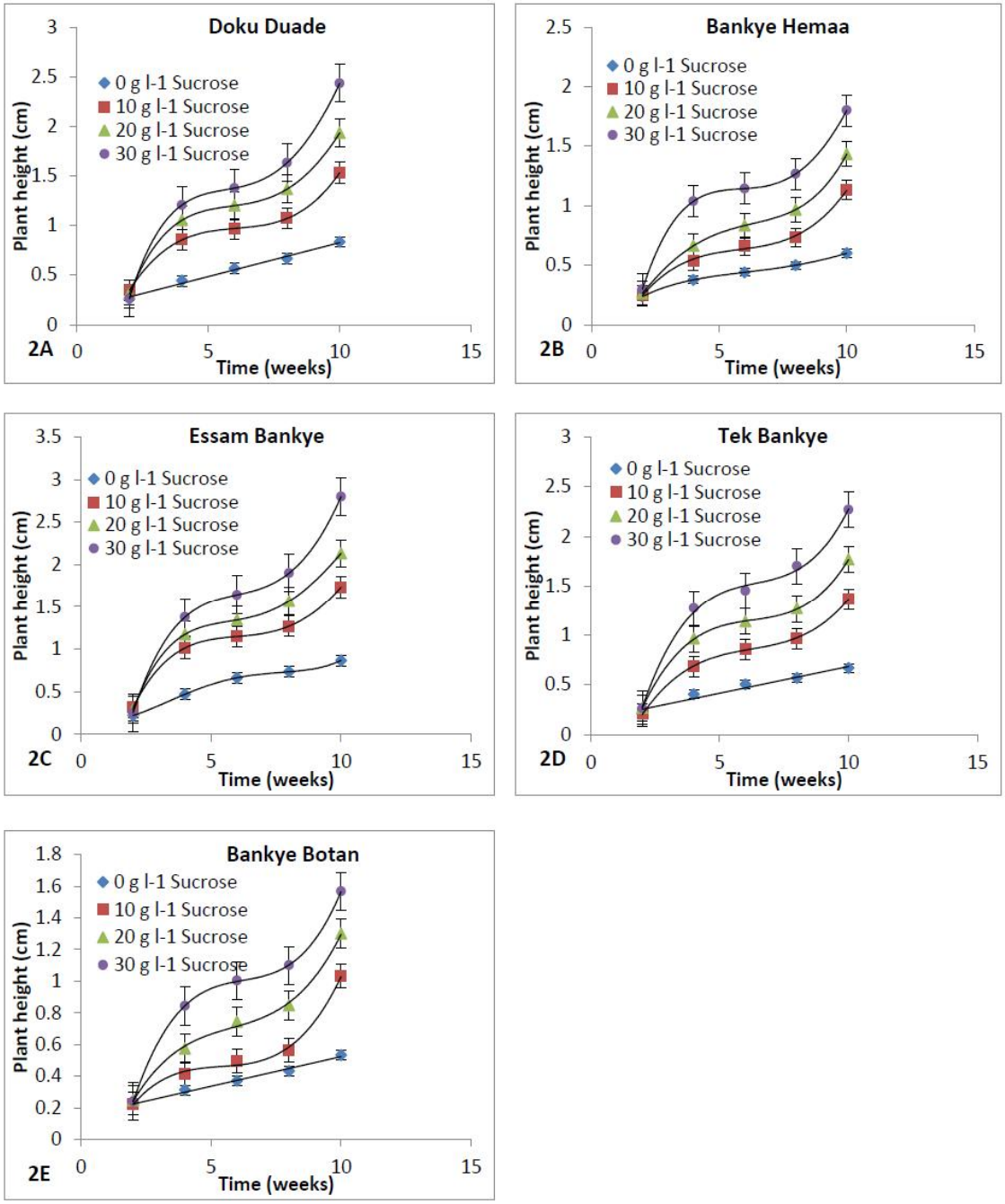


**Fig. 2. Sucrose and time effects on plant height of cassava genotypes cultured *in-vitro* using apical meristem in hormone free Murashige and Skoog basal media. Response curves were determined by orthogonal polynomial contrast analysis determined at *P=.05***

Sub-cultured genotypes using the recommended sucrose levels showed slow growth process and remained healthy even after they were cultured for seven months. However, senescence of leaves was observed after eight months. All genotypes, with the exception of Bankye Botan, outgrew the test tubes after they were sub-cultured for eight months (Fig. 4).

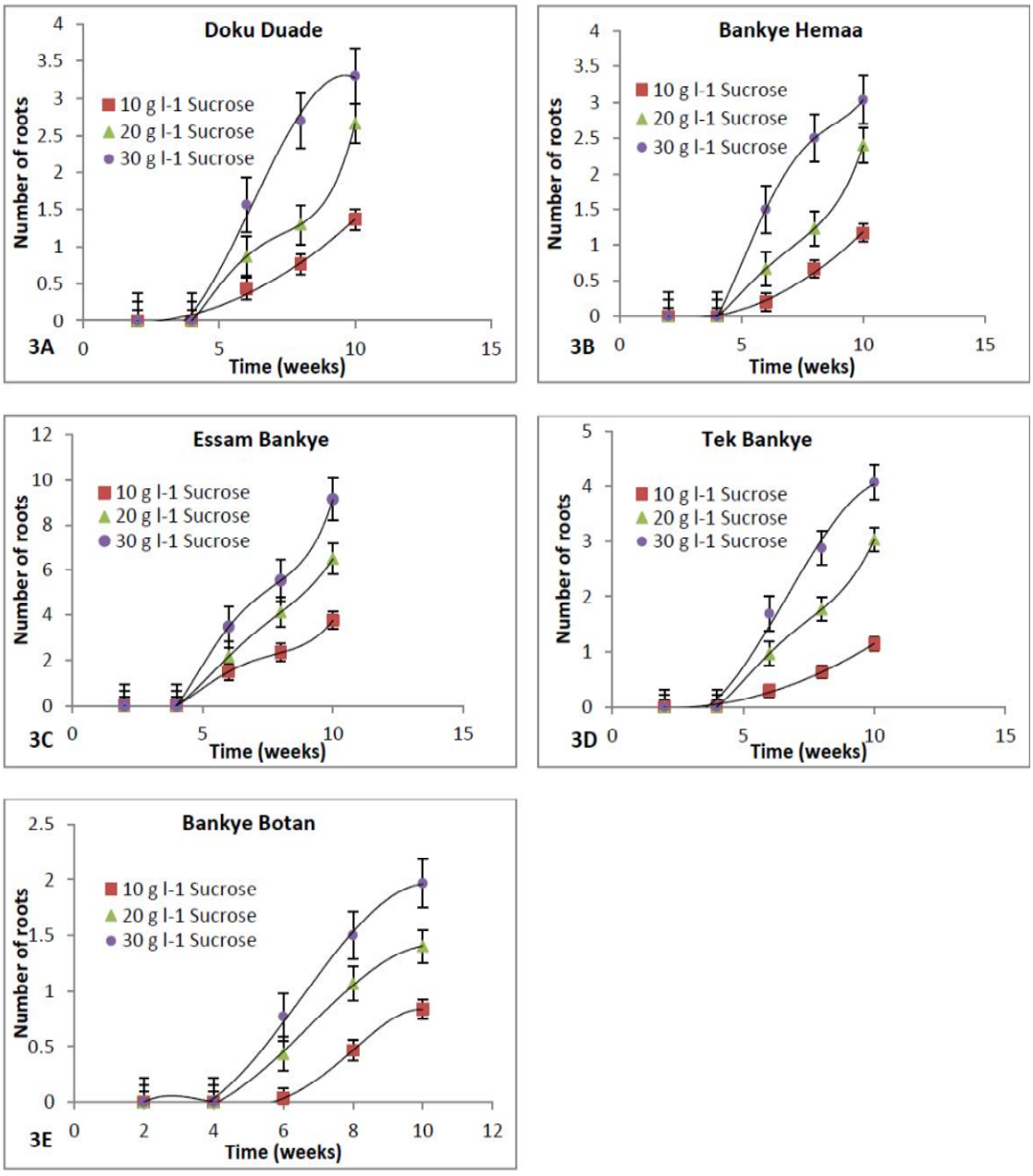


**Fig. 3. Sucrose and time effects on root formation of the cassava genotypes cultured *in-vitro* using apical meristem in hormone free Murashige and Skoog basal media. Response curves were determined by orthogonal polynomial contrast analysis determined at *P=.05***


**Fig. 4. Sub-cultured genotypes, using recommended sucrose levels, after culturing for eight months using 2-node stem cuttings in hormone free Murashige and Skoog basal media**
*†Essam Bankye (10 g $l^{-1}$ sucrose); ‡Doku Duade (20 g $l^{-1}$ sucrose); §Tek Bankye (20 g $l^{-1}$ sucrose); ¶Bankye Hemaa (30 g $l^{-1}$ sucrose); #Bankye Botan (30 g $l^{-1}$ sucrose)*

## 4. CONCLUSION

The cassava genotypes used in this study showed a direct regeneration without callus formation when cultured in hormone free MS basal medium using meristem tips. Generally, sucrose enhanced the growth performance of plantlets; however, the genotypes responded differently to sucrose in leaf formation, plant height, and rooting ability with time. This study has demonstrated that reduction of sucrose in hormone free MS basal medium can be used to induce slow growth in Essam Bankye, Doku Duade, and Tek Bankye, without a significant impact on the health of the plantlets. Thus, sucrose levels of 10 g $l^{-1}$ for Essam Bankye, 20 g $l^{-1}$ for Doku Duade and Tek Bankye, and 30 g $l^{-1}$ for Bankye Hemaa and Bankye Botan were the recommended rates for *in-vitro* conservation.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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