EVALUATING IN-VITRO REGENERATION POTENTIAL FOR HIGH BETA-CAROTENE CASSAVA (*Manihot esculenta)* CLONES

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Abstract
In-vitro regeneration potential for three high beta-carotene (pro-vitamin A) cassava varieties - UMUCASS 36, UMUCASS 37 and UMUCASS 38, and a control variety TMS 60444 were evaluated and optimized as a preliminary step towards the introgression of more nutritional and agronomic traits. Somatic embryos developed from *in vitro* plantlets were used for the production of callus tissues from which whole cassava plantlets were regenerated. The frequency of somatic embryogenesis (SE) for UMUCASS 36, 37 and 38 were 43% (10/23), 50% (8/16) and 43% (20/47) respectively while that for the TMS 60444 used as control was 64% (38/59). The regeneration efficiency expressed as the percentage of plant lines recovered from total number of cotyledon lines derive from UMUCASS 36, 37, 38 and TMS 60444 were 40% (2/5), 29% (2/7), 38% (3/8) and 67% (10/15) respectively. The regeneration efficiencies for the UMUCASS varieties were less than the control and thus require further optimization of the regeneration protocol for bulking of regenerated plantlets derived from these genotypes.

Keywords: UMUCASS Varieties, Regeneration and Agronomic Traits

Introduction

Cassava is a tropical, woody shrub which originated from South America. It is cultivated throughout the world for its tuberized, starchy roots. It is one of the main sources of dietary carbohydrate after rice and maize in the tropics. It is consumed by over 800 million people (Liu *et al*., 2011). Cassava is grown as a subsistent as well as a food security crop in Nigeria due to its adaptive nature to adverse environmental conditions such as drought, low nutrient concentration and infertile, acid soils (Zainuddin *et al.,* 2012; Nyaboga *et al*., 2013).

Although cassava tuberous roots are high in caloric content and in major nutrients such as calcium and phosphorus, it is highly deficient in protein, vitamin A and essential micronutrients such as iron and zinc (Sayre *et al*., 2011). Populations depending on cassava as a staple are at risk of suffering from micronutrient deficiency commonly referred to as hidden hunger, and are often limited by resources to supplement their diet with food supplements or food diversification. Aside from nutrient deficiency, cassava is susceptible to a host of bacterial and viral diseases including cassava bacterial blight (CBB), cassava mosaic disease (CMD) (Legg *et al*., 2015), cassava brown steak disease (CBSD) (Ephraim *et al*., 2015) and host of others. It also suffers from pest and insect infestations, particularly the whiteflies and aphids that serve as vehicles for most viral diseases. Other factors limiting cassava utilization include short shelf-life as a result of rapid deterioration of the roots two days after harvest due to post-harvest physiological deterioration, and the presence of cyanogenic glucoside which could cause death if not well-processed cassava roots are consumed (Akinpelu *et al.,* 2011). Constraints to cassava varietal development which include high degree of heterozygozity, low pollen fertility and inconsistent flowering (Liu *et al*., 2011) also limit the use of conventional breeding approach to solve identified cassava production, storage, processing and utilization constraints.

The recent achievement in breeding for high beta-carotene cassava as a means of alleviating vitamin A deficiency mostly in women and children (Akinwale *et al*., 2010) provides a platform for the introgression of other essential nutrients such as zinc and iron to further enhance the nutritional

content of the crop. It is also important to improve these newly developed high β-carotene genotypes for other agronomic traits such as pest and disease resistance (Alabi *et al.,* 2011; Bellotti *et al.,* 2012), tolerance to post-harvest physiological deterioration (PPD) and elimination of cyanogenic glycosides (Akinpelu *et al.,* 2011). Although lots of conventional breeding efforts are being invested in solving these problems, genetic modification through transgenesis provides additional tools that help breeders develop improved varieties of root and tuber crops more effectively and efficiently (Liu *et al.,* 2011; Sayre *et al.,* 2011). Target tissue for genetic modification is an essential component of transgenic technology system, and one of the factors that determine the success of developing improved varieties through genetic engineering. Cassava transformation is genotype dependent and requires the establishment of regeneration protocol for each cultivar to determine its transformability. In this study, we evaluated the transformability of three high beta-carotene varieties; UMUCASS 36, UMUCASS 37 and UMUCASS 38 as a preliminary step to introducing other essential agronomic and nutritional quality traits into these varieties.

Materials and Methods

Somatic embryogenesis

Immature leaf lobes from 4-weeks-old *in-vitro* propagated cassava cultivars - TMS 60444, UMUCASS 36, UMUCASS 37 and UMUCASS 38 were cultured on DKW/Juglans medium supplemented with 20% sucrose, 50 µM picloram (DKW2 50P) and 7 g of Agar for the induction of somatic embryos. The culture plates were incubated in the growth chamber for a period of 4 weeks at 28^0C under dark regimen (Taylor *et al*., 2012). The development of somatic embryos termed organized embryogenic structure (OES) was monitored bi-weekly using dissecting microscope. Quality of the OES was determined by yellow solid structures developing from the midrib of the cultured leaf lobes. The developing somatic embryos were multiplied by sub-culturing them bi-weekly thrice to generate adequate quantity to initiate the development of the friable embryogenic callus phase. Efficiency of embryogenesis was determined by the number of leaf lobes that produced somatic embryos.

Friable callus induction

Developed somatic embryos were excised with hypodermal needle and squashed with forceps before being transferred into a freshly prepared Gresshoff and Doy medium supplemented with 20% sucrose , 50 µM of picloram (GD2 50P) and 7 g of Agar. The squashed embryos were transferred as colonies onto the GD2 50P medium to aid callus induction. The culture plates were incubated for another 4 weeks in the growth chamber with the same parameters as stated above. Embryogenic callus induction was also monitored with dissecting microscope and quality callus lines were determined by whitish friable tissues termed friable embryogenic callus (FEC) growing from the squashed somatic embryos.

Plant regeneration

Friable Embryogenic Callus (FEC) tissues were cultured firstly onto Murashige and Skoog medium supplemented with 30 % sucrose, 7 g of Agar (MS3-agar) and 0.5 µM of naphthalene acetic acid (NAA) referred to as Stage 1 regeneration medium for the maturation and development of somatic embryo (Okwuonu *et al*., 2015). After a period of 3 weeks, the maturing somatic embryos were transferred to a new medium composed of MS3-agar and 0.05 µM of NAA for cotyledon development. After another 3 weeks incubation period, the developing cotyledon was transferred to a shoot regeneration medium composed of MS3-agar and 2 µM of benzylaminopurine (BAP) for shoot regeneration. Finally, the regenerated shoot were transferred to rooting medium (MS3-agar without supplements) for rooting and regeneration of whole cassava plantlet. The regeneration efficiency was determined by the number of plant lines derived from the total number of cotyledon lines regenerated.

Results and Discussion

Production of somatic embryos and callus induction

Vigorous and healthy somatic embryos determined by the development of solid yellow structures from the midrib of cultured leaf lobes were produced from the three high beta-carotene clones: UMUCASS 35, UMUCASS 36 and UMUCASS 37 in comparison to the model cassava cultivar TMS 60444 used as control in the experiment (Fig. 1). The embryogenic mass of UMUCASS 35, UMUCASS 36, and UMUCASS 37 consisted more of undifferentiated cells in the form of mushy-like substances and less of the solid yellow structure. Table 1 shows the number of OES derived from established leaf lobes as well as the efficiency of embryogenesis determined by the percentage value of the number of OES formed from the number of leaf lobes established. The efficiency of embryogenesis of the model cassava cultivar TMS 60444 with optimized protocol for OES induction gave the highest percentage value of 64% (38/59) compared to the high β-carotene cultivars. The UMUCASS varieties under evaluation gave very similar results of 50% for UMUCASS 36 and UMUCASS 37, and 43% for UMUCASS 38. The means of somatic embryos produced from the four cultivars showed that while the quantity of somatic embryos produced by TMS 60444 (the control) was significantly ($p<0.05$) higher than the three cultivars tested, there is no significant difference among the means of the pro-vitamin A cassava cultivars.

Table 1: Efficiency of somatic embryogenesis

Cultivar	Total number of leaf lobes established	Number of OES formed	Percent OES formed
TMS 60444	59	38	64
UMUCASS 36	23	10	43
UMUCASS 37	16	8	50
UMUCASS 38	47	20	43

Callus induction was determined by the production of FEC tissues on the surface of the OES. These callus tissues were observed in the three UMUCASS varieties as well as in the control variety for their growth attributes. Profusely and vigorously growing FEC tissues were produced from TMS 60444 while moderately growing callus tissues were produce by the UMUCASS varieties (Figure 1). This result shows that the three UMUCASS varieties evaluated in this experiment have great potentials for producing high quality embryogenic callus tissues but will require further optimization to produce larger quantities of this tissue.

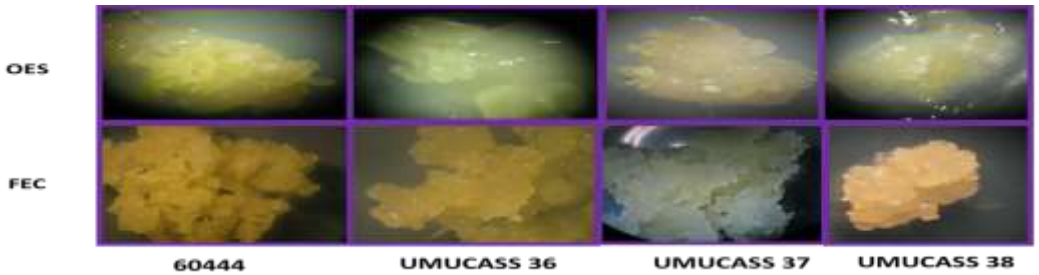

Fig.1: Production of somatic embryo and embryogenic callus induction from *in-vitro* propagated mother plant of TMS 60444, UMUCASS 36, UMUCASS 37 and UMUCASS 38

Table 2 shows the number of somatic embryos regenerated, number of cotyledons regenerated, number of plantlets recovered, and the percent plant recovered. All four cultivars produced cotyledons from embryogenic callus tissues. However with subsequent transfer of embryogenic tissues to Stage 2 regenerating medium consisting of MS3-agar supplemented with 0.05 µM NAA, the control genotype TMS 60444 produced more somatic embryos (2 to 3 times) more than the three UMUCASS genotypes after 21 days incubation on the new medium (Table 2). When the funnel-shaped embryos observed on Stage 2 regeneration medium were subsequently transferred to germination medium consisting of MS3-agar supplemented 2 µM BAP for shoot regeneration,

cotyledons were regenerated. The numbers of regenerated cotyledon lines are shown in Table 2. The control genotype TMS 60444 produced two to three times (15) the number of cotyledon lines produced by the three UMUCASS genotypes (5 to 8). Similar results were obtained with the transfer of the regenerated roots to root inducing medium. The regeneration efficiency determined by the percentage value of the number of plant lines derived from the regenerated cotyledonous embryo lines were recorded as 40% (2/5), 29% (2/7) and 38% (3/8) for UMUCASS 36, 37 and 38, respectively compared to 67% (10/15) regenerated from TMS 60444.

Table 2: Regeneration efficiency of cassava genotypes evaluated

Cultivar	No. somatic embryos regenerated	No. of cotyledon regenerated	No. of plantlets recovered	Percent plant recovered
TMS 60444	23	15	10	67
UMUCASS 36	8	5	2	40
UMUCASS 37	8	7	2	29
UMUCASS 38	12	8	3	38

Results obtained from this study showed that the frequency of somatic embryogenesis and regeneration efficiency of UMUCASS varieties were less compared to the model cassava cultivar TMS 60444. This is indicative of the fact that somatic embryogenesis and organogenesis of cassava is genotype dependent and thus requires that elite genotypes are evaluated for such attributes. This result agrees with the findings of Ravindran *et al.* (2014) who evaluated the influence of age of explant and genotype on the somatic embryogenesis of some African and Indian cassava varieties. Their study showed that production of somatic embryos was highly dependent on cultivar used at $p<0.0001$. Also Monogomake *et al*. (2015) in their study on the somatic embryogenesis and organogenesis of cassava landraces from Cameroon confirmed that shoot bud induction from green cotyledon varied across cultivars under investigation. It was imperative to evaluate the frequency of somatic embryogenesis and regeneration of this very important pro-vitamin A cassava for subsequent introgression of important agronomic and nutritional traits through transgenesis.

The optimized protocol for the model cassava cultivar TMS 60444 was adopted for this study to serve as baseline protocol for subsequent optimization of the regeneration protocol for UMUCASS varieties. Studies have shown that the essential factors to consider in cassava somatic embryogenesis and organogenesis include explant type and age (Ravindran *et al*., 2014; Opabode *et al.* 2016), media type (Anuradha and Balasubramanina 2015), growth regulatory hormone (Li *et al*., 2012) and method of manipulation (Nyaboga *et al*., 2015). Here we used DKW 50P for OES induction and GD2 50P for FEC induction because Picloram at 50 µM has been confirmed as the most effective and efficient hormone for the production of high quantity and quality cassava somatic embryo from young tissues (Ravindran *et al.*, 2014). Despite the high frequency of somatic embryogenesis achieved with this concentration of Picloram, Mongomake *et al*. (2015) has shown that different Cameroon landraces, Ngan Mbada and Ekona red performed differently at 50 µM Picloram with Ngan Mbada giving a SE frequency of 40% and average number of somatic embryos of 90 and Ekona red showing SE frequency of 47% and average number of somatic embyros of 45. This result is similar to the data derived from our study thereby suggesting that variation in concentration of picloram could improve the frequency of somatic embryogenesis in the UMUCASS varieties.

Immature leaf lobes were used as explant type being wildly adopted by the cassava community as the desired explant for OES induction. However, Ravindran *et al*. (2015) has shown that explant type influences the induction of somatic embryos in different cassava varieties. Two Indian landraces, H165 and Kibaha and three African cultivars 96/1089A, TME3 and TME4 performed

best with immature leaf lobes while TMS 30572 performed best with axillary bud. This suggests that axillary bud could be a better explant type for OES induction in most other cassava varieties and could be evaluated with the UMUCASS variety.

This study is important in illustrating the regeneration potentials of UMUCASS 36, UMUCASS 37 and UMUCASS 38 thus establishing a platform for the genetic transformation of these important pro-vitamin A cassava for improved agronomic and nutritive traits. However, Successful production of transgenic events in numbers adequate for molecular, field and regulatory evaluations require an efficient regeneration protocol for the desired cultivar. There is need to optimize the regeneration protocol of the UMUCASS cassava variety by manipulating the different factors essential for somatic embryogenesis and organogenesis.

Conclusion

Good quality OES that generated FEC target tissues for transformation were obtained from UMUCASS 36, UMUCASS 37 and UMUCASS 38, thereby establishing a transformation platform for the introgression of more agronomic traits of interest. However, the efficiency of regeneration of the three UMUCASS genotypes was lower compared to TMS 60444 used as control in the experiment. There is need for further optimization of the regeneration protocol to enhance the number of plantlets recovered from the high beta-carotene genotypes thus increasing the selection pool for a quality transgenic event.

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