Influence of Age of Explants and Genotype on Somatic Embryogenesis in African and Indian Cassava Cultivars

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Abstract

Potential of eight cassava cultivars viz., TME3, TME4, TMS 30572, TMS 1089A, 96/0160, Albert, Kibaha and H165 for initiating somatic embryos was studied. Two different explants, young leaf lobe pre-cultured on Murashige and Skoog (MS) medium and axillary bud pre-cultured on MS medium containing BAP were tried. The age of explants and type of cultivar highly influenced the frequency and quantity of somatic embryos. Axillary buds of cv. Albert and young leaf lobes of cv. H165 responded well, while the same results were observed for conversion and development. Hardening and establishment in green house was close to 100% for all the cultivars under study. This paper discusses the effect of juvenility of the explants used, duration of pre-treatments and culture on embryogenesis medium, cultivar and its preference of explant, health of the mother plants, on successful regeneration of cassava via somatic embryogenesis, which could be very resourceful for friable embryogenic calli production and successful genetic transformation.

Key words: African cultivar, cassava, embryogenic callus, somatic embryos

Introduction

Cassava (*Manihot esculenta* Crantz) was introduced to East Africa from Brazil by Portuguese in the 17th century then to India, Java and South East Asia by 18th century (Byrne, 1984). Its storage root has the maximum starch storage approaching the known physical limit of 30%. Though many other starchy crops and fruits were introduced at the same period to the developing countries in the tropics, cassava remained a steady and abundant supplier of food throughout the year even during the times of natural disasters and war. This crop has unique place in global food system owing to its capacity to produce more food per unit area, ability to withstand adverse environmental and climatic conditions, flexibility to be harvested at convenience, which made it as a popular crop in the developing countries. Apart from being a staple food, cassava is very versatile and its starch and derivatives are used to develop a variety of products such as value added foods, confectionery, sweeteners, glues, plywood, textiles, paper, biodegradable products, monosodium glutamate, and drugs (Gondwe et al., 2001; Chitundu et al., 2006; Haggblade and Nyembe, 2007). Cassava chips and pellets are used in animal feed and alcohol production. Cassava leaves are also a very good source for bio-pesticide and bio-fumigant (CTCRI, 2011).

Cassava is the only edible species belonging to the family *Euphorbiaceae* which is inherently heterozygous in nature. Though cassava grows on varied environmental and climatic conditions, it is susceptible to invading pests and pathogens like whitefly, mealy bug, green mite, cassava mosaic viruses and cassava brown streak viruses. The white fly also serves as a vector for viral diseases. Cassava mosaic disease could be very serious if proper care is not taken. Indian cassava mosaic virus in India and African

cassava mosaic virus in Africa could cause up to 40 and 95% yield loss, respectively (Edison et al., 2006, Bocks and Woods, 1983). In the recent years, there has been increased emphasis on optimizing agricultural production in conjunction with conserving the natural resources through improved crop and crop management systems. Efforts were made to improve the crop's agronomic traits via conventional breeding that allows the combination of favorable traits from different species and has been used successfully to produce few resistant varieties, nevertheless these efforts were largely hindered by in-breeding depression and the polygenic and recessive nature of many desirable traits (Makwarela and Rey, 2006). Moreover, inter-specific hybridization program can be slow and require a great deal of scientific expertise and skilled labor. Genetic engineering has been identified as a powerful tool to overcome the limitations of conventional breeding by allowing the introduction of desirable traits directly into farmer preferred cultivars (Thro et al., 1999). *Agrobacterium*- mediated transformation via somatic embryogenesis or friable embryogenic calli (FEC) derived from them have been recognized as the best way for achieving successful transformation events (Raemakers et al., 1997; Schopke et al., 1998; Munyikwa et al., 1998; Taylor et al., 2001; Raemakers et al., 2001; Hankoua et al., 2006; Vanderschuren et al., 2007; Bull et al., 2009; Liu et al., 2011; Putten et al., 2012; Taylor et al., 2012).

Since three decades, cassava regeneration via somatic embryos on various explants had been tried and improved. Somatic embryos had been induced on the embryonic axes (Stamp and Henshaw, 1982; Konan et al., 1994), young leaf lobes (Stamp and Henshaw, 1987; Szabados et al., 1987; Raemakers et al., 1993; Sofiari et al., 1997; Mussio et al., 1998; Li et al., 1998; Raemakers et al., 2000), cotyledons (Stamp and Henshaw, 1987a), shoot tip explants (Ogburia, 2003) and from somatic embryo explants (Guohua, 1998; Groll et al., 2001). Previously somatic embryogenesis on cassava was attempted using auxins such as 2,4-D, or/and NAA (Szabados et al., 1987; Sofiari et al., 1997; Guohua, 1998; Li et al., 1998; Guohua and Qiusheng, 2002). It was a little more than a decade ago, the potential of picloram in the medium for better efficiency of cassava somatic embryogenesis was recognized (Zhang et al., 2000). This trial was followed by Zhang et al. (2001), Taylor et al. (2001), Hankoua et al. (2005), Zhang and Puonti Kaerlas (2005), Saelim et al. (2006), Hankoua et al. (2006), Atehnkeng et al. (2006), and Feitosa et al. (2007). All these research teams found that picloram at $50\,\mu$M was the most effective and efficient in producing high quality and quantity of somatic embryos from juvenile explants of cassava. Same plant growth regulator (PGR) was competent to produce high frequency transformation events as well (Bull et al., 2009; Liu et al., 2011; Putten et al., 2012; Taylor et al., 2012). High quality repeatable somatic embryogenesis is an essential prerequisite for FEC production and thereby efficient transformation. In this paper, the effectiveness of picloram at $50\,\mu$M in somatic embryogenesis media on different African cassava cultivars in comparison with a popular Indian cultivar for somatic embryogenesis has been analyzed using two different explants.

Materials and Methods

Plant materials

African cassava cultivars TME3, TME4, TMS 30572, TMS 1089A, 96/0160, Albert, and Kibaha and a popular Indian cultivar H165 were maintained on MS medium (Murashige and Skoog, 1962) with half strength ammonium nitrate and potassium nitrate (MS Mod.3B, Duchefa, The Netherlands) containing 2% sucrose, pH adjusted to 5.8 and solidified with 0.75% plant agar (Duchefa). All the cultivars were indexed for the absence of viruses. Nodes and shoot tips of these mother plantlets were sub-cultured every 6 to 8 weeks and every 12 months, new mother plants were raised from nodes taken from the green-house-grown plants.

Explant harvest

Two treatments were used for explant initiation. In the treatment 1, very young unopened leaf lobes were excised carefully from the nodes cultured upright on MS medium supplemented with $2.0\,\mu$M $CuSO_4$ (MSC). Under treatment 2, enlarged axillary buds were surgically taken from the nodes placed horizontally over cassava axillary bud induction medium (CAM) (Bull et al., 2009). All the cultures and mother plants were maintained at 25 ± 2°C under 16/8 h photoperiod provided by cool fluorescent tube lights (2500 to 3000 lux).

Somatic embryogenesis

The harvested explants were cultured on MS medium augmented with $2.0\,\mu$M $CuSO_4$ and $50\,\mu$M picloram

(MSCP) and were maintained at 25 ± 2°C under dark. Development of embryogenic calli and somatic embryos were recorded every 21 days when the embryogenic calli were sub-cultured onto MS medium containing 1.0 μM NAA and 2.0 μM BAP (MSNB) for embryo maturation. Embryo maturation was allowed at the normal culture room conditions as the mother plant cultures. While recording the observations, the explants that developed somatic embryos alone were considered as responded.

Acclimatization

After maturation, the rooted young plantlets were transferred after thorough washing under running tap water to small pots of 4 inch width containing sterile vermiculite and were maintained under culture room conditions. They were kept covered using punctured transparent polythene bags to provide humidity. They were irrigated once a week using Hoagland's solution (Hoagland and Arnon, 1938). When the plants reached a height of 5-6 inches, and appear healthy, they were replanted into bigger pots containing sand: soil: manure mixture (1:1:1) and were transferred to glass house.

Statistical analysis

Data on efficiency of somatic embryogenesis from axillary bud and leaf lobe explants of eight cassava cultivars on MSCP medium were analysed through one-way Analysis of variance (ANOVA). When significant differences were detected (P < 0.05), the Tukey's Honestly Significant Difference (HSD) multiple comparison test was applied to compare the cultivar level differences. All the statistical analyses were carried out using statistics software SPSS (SPSS software for Windows release 10.0; SPSS Inc., Chicago, IL).

Results and Discussion

Influence of explants on somatic embryogenesis

Type of plant and developmental stage of explant are very important in inducing somatic embryo development. The leaf lobes more than 5-6 mm in length didn't produce embryogenic calli. On leaf lobe explants, callus development was initiated at the petiole region within 5 days of culture on MSCP medium and doubled in 15 days' time and somatic embryos started appearing as globular structures. Placing the explants with the adaxial side or the abaxial side touching the medium, did not affect somatic embryogenesis. Though callus initiation occurred at the petiole or cut region, within 5 to 10 days the callus proliferated from the entire explant. If the leaf lobe explants

were more than 5-6 mm in length, or older than 2-3 days, they did not or rarely produced somatic embryos. Instead, they produced hard watery callus, sometimes cottony white, which turned brown in 15-20 days and died. These results are consistent with Szabados et al. (1987), who reported the inefficiency of larger or older leaf lobes in developing somatic embryos. Alternatively, Li et al. (1998) recommended to use the juvenile material for higher capacity of shoot regeneration while immature leaf lobes and axillary buds for producing either primary or secondary somatic embryos was suggested by several past works (Sofiari et al., 1997; Guohua, 1998; Groll et al., 2001; Taylor et al., 2001; Zhang et al., 2001; Zhang and Puonti-Kaerlas, 2005; and Taylor et al., 2012).

While excising the axillary bud, care must be taken to avoid scraping the stem tissues. Once the stem tissues were scraped along with the axillary bud, they produced cottony white callus which over grew the embryogenic callus developed from the bud. This was true for all the cassava cultivars under study. Within 15 to 20 days, the calli started developing organized globular structures and initiated somatic embryogenesis. Cottony white callus was often found on axillary bud explants, however it did not affect the somatic embryo development.

Plant growth regulations play a pivotal role in tissue de-differentiation and re-differentiation. It also depends on the endogenous hormone levels and their synergistic/antagonistic action with external PGRs. The amount of callus formed on axillary bud explants were more than the calli produced using leaf lobe explants. The axillary buds were made to enlarge on BAP containing media before it was placed on MSCP for calli and subsequent somatic embryo induction. The BAP pre-treatment of the axillary buds might have triggered the callus induction. Szabados et al. (1987) also observed that callus formation was more intense on media with higher BAP concentration.

Two explants used in this work were equally effective but on different cultivars. When H165, Kibaha, 96/1089A, TME3 and TME4 preferred young leaf lobes; TMS 30572 best responded on axillary bud with an acceptable percentage of response. Axillary bud and leaf lobe explants served the cultivars Albert and TMS

96/0160 almost equally well towards frequency and efficiency of somatic embryo production. Leaf lobe explants produced less amount of calli compared to that of axillary bud explants in all the experiments and for all the cultivars under study (Table 1). There was no relation between amount of calli produced and the number of somatic embryos developed (Table 1).

Frequency of somatic embryo development

Response of number of explants per experiment was highly variable between the treatments and among the cultivars. Cassava cv. H165 showed a high affinity towards young leaf lobe explants to produce somatic embryos reaching almost 100%. Among the African cultivars Albert and TMS 96/0160 showed higher frequency as compared to others. Both Kibaha and 96/1089A demonstrated good response with axillary bud explants and young leaf lobe explants, respectively (Fig. 1).

Duration of the cultures on MSCP medium was crucial to attain maximum efficiency. The leaf lobes on MSCP medium had to be sub-cultured within 21 to 25 days. If it was retained on the same medium for longer, the callus started browning and the embryogenic capability reduced significantly (data not given). After 35 to 40 days of retention on the same medium, the callus died. This duration also slightly varied from cultivar to cultivar. Mussio et al. (1998) also had observed that when the explants were placed on induction medium for more than 10 days it delayed shoot formation and more than 20 days on pre-culture medium reduced the somatic embryogenesis efficiency by almost 70% (Guohua, 1998).
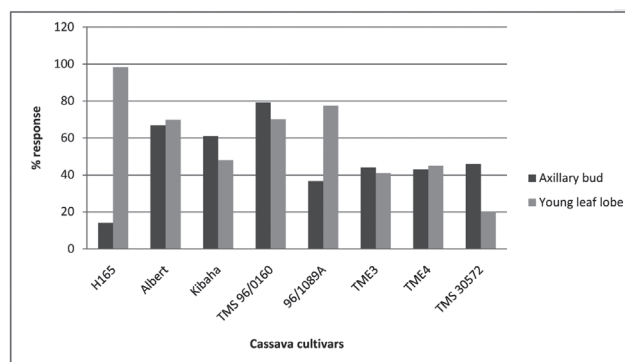

Fig. 1: Frequency of different cassava cultivars in producing somatic embryos on axillary bud and leaf lobe explants when cultured on MSCP medium.

Most of the successful transformation protocols for cassava also indicate exact time duration for sub-cultures to obtain optimum results (Raemakers et al., 1997; Taylor et al., 2001; Vanderschueren et al., 2007; Bull et al., 2009). Sofiari et al. (1997) found that continuous and long term use of either 2,4-D or NAA in embryo induction medium causes it to shift from embryogenesis to root formation and that this effect could be reversed by using the PGRs alternatively. This shows that external PGR application considerably alter the hormonal composition and concentration on organ development. Somatic embryogenesis is a complex process and it needs precise hormonal combinations for a cell to become bipolar and to divide to form a complete organism. The external PGRs we provide gains important attention in determining the fate of the tissue. It is also highly dependent on the genetic makeup and the endogenous hormone levels of the explant and that is explicitly why

Table 1: Data on efficiency of somatic embryogenesis from axillary bud and leaf lobe explants of eight cassava cultivars on MSCP medium.

Cultivar	Somatic embryos developed		Amount of callus induced	
	Axillary bud	Young leaf lobe	Axillary bud	Young leaf lobe
H165	35.3 ± 1.3*	38.88 ± 2.625[a**]	+++	++
Albert	39.5 ± 1.6	26.56 ± 11.13[a]	++++	+
Kibaha	10.21 ± 2.33	28.6 ± 2.66[b]	++	++
TMS 96/0160	30.3 ± 2.21	10.5 ± 3.8[b]	++	+
96/1089A	17.6 ± 1.3	32.3 ± 3.33[b]	++	+
TME3	4.5 ± 1.5	18.33 ± 2.5[c]	+	+
TME4	3.8 ± 2.1	19.86 ± 1.37[c]	+	+
TMS 30572	27.8 ± 8.5	16.2 ± 1.66[b]	++	++

+ = 50 mg calli

* Values are expressed as mean ± standard deviation.

**Numbers indicated using same alphabets were not significantly different from each other.

there is a need to standardize the protocols for different cultivars separately.

Cultivar and the explants used were important determining factors for somatic embryo production. In our studies at 5% confidence level, the emergence of somatic embryos highly depended on the cultivar used (p = < 0.0001). The response of leaf lobe explants in cultivars H165 and 96/1089A were very high when compared to other cultivars and axillary bud explants. However, axillary bud explants produced significant results for cultivars Albert, 96/0160 and Kibaha. These cultivars were responding equally well on leaf lobe explants as well, except for Kibaha (Table 1). The response of TME3, TME4, and TMS 30572 was generally low compared to other cultivars, but they kept a similar response pattern for both the explants tried unlike other cultivars. Significance of the differences found were identified using Post hoc and Tukey HSD test and is indicated in the table as sub-sets. These results indicate that it was very important to standardize the explant material for each cultivar to obtain a maximum output under a given condition.

Quality and quantity of somatic embryos produced and their conversion to plantlets

Number of somatic embryos developed per explant was manually counted under a light microscope to determine the efficiency. The appearance of the somatic embryos, rooting ability, and its conversion into a plantlet was considered while determining its quality. Though H165 showed high frequency and efficiency for somatic embryo production, the embryos developed were more tubular and thin. However, once sub-cultured on MSNB medium for embryo maturation, the embryos looked normal and healthy. African cultivars were also efficient in producing somatic embryos except for cultivars TME3, TME4 and TMS 30572 as compared to others. Subculturing either to MSCP or to MSNB within 30 days is a must for the embryos to

develop and mature. Somatic embryos from the African cultivars showed similar vigor in growth and conversion. The quality of somatic embryos was better when the leaf lobes or axillary buds were used from healthy and vigorously growing mother plants. Recycling mother plants *in vitro* for a long time reduced the growth vigor and they started appearing thin and fragile. Re-establishing the mother plants every 12 months from green-house-grown plants helped in overcoming this problem.

Maturation and rooting of the somatic embryos occurred in the same medium (MSNB) for cultivars H226, H165, and Albert but other cultivars had to be sub-cultured onto basal MS medium without PGRs for root development after the embryos develop cotyledons completely. Rooting initiated within 5 days of culture. Most of the embryos developed a strong single root but when the primary root is damaged before sub-culturing to MSC medium, more than one root was developed from the base. Well rooted plantlets were hardened and after three to four weeks or when new leaves are formed, and reached 4-5 inches in height, they were transferred to bigger pots and all the plants were successfully established in the greenhouse conditions (Fig. 2). Sofiari et al. (1997), Raemakers et al. (2000), and Groll et al. (2001) desiccated the embryos before rooting for homogenous response, but in our studies rooting was initiated without a desiccation phase being provided. Ninety percentage of the plantlets transferred to sterile vermiculite survived and if the
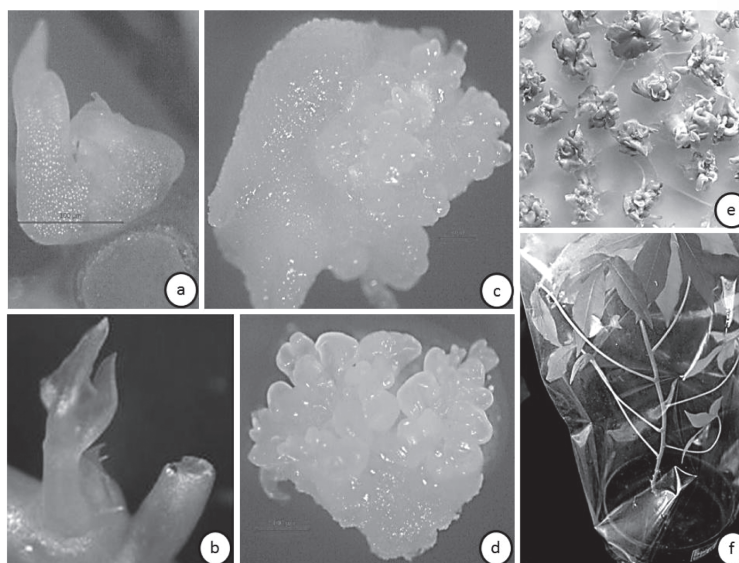

Fig. 2: Somatic embryogenesis, embryo maturation and acclimatization in cassava cultivars. a) and b) leaf lobe and axillary bud explants, respectively c) and d) somatic embryos developed on MSCP medium on cultivars H165 and Albert, respectively e) embryo maturation and rooting on MSNB medium f) plantlet ready for transfer to green house after acclimatization (scale in the figure indicates 100 μm)

roots were not disturbed, 100% of the plantlets transferred to bigger pots containing soil: sand: manure (1:1:1) survived, acclimatized and established themselves in green house conditions.

Conclusion

Somatic embryogenesis is an inevitable tool for producing true to type next generation of cassava, genetic manipulation studies and germplasm maintenance. Cassava transformation has been a difficult task since decades. It is mostly due to lack of dexterous repeatable protocols for somatic embryo production. From our studies we infer that juvenility of the explants used, duration of pre-treatments and culture on embryogenesis medium, cultivar and its preference of explant, health of the mother plants used, all mattered in the successful and repeatable high efficiency somatic embryo production. These results could be a base for production of cotyledon explants or FEC generation for genetic transformations of these cultivars, for disease and pest resistance and quality improvement via genetic engineering.

Acknowledgement

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