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Exploring the African cassava (*Manihot esculenta Crantz)* germplasm for somatic embryogenic competence

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Somatic-embryogenic competence of eleven cassava genotypes was determined in induction media containing 8 and 12 mg/l of the auxin picloram, using axillary meristems and leaf lobes as explants. There were significant differences (p<0.01) among the cassava genotypes for ability to form somatic embryos. Proembryo formation took between 27 to 35 days and ranged between 34.5% for TME 596 and 0% for SL80/40. Embryo formation at the two picloram concentrations were not significantly different. Pro-embryos formed by two genotypes viz I96/1439 and I95/0528 did not survive beyond the globular developmental stage. Generally the level of proembryo formation was not adequate as an indicator of embryogenic competence. Development to torpedo stage took between 55 to 65 days. Higher success was achieved with leaf lobes (21.7%) than axillary meristem (13.8%). There was a significant (p<0.01) genotype x explant interaction, indicating that ability of the cassava genotypes to undergo somatic embryogenesis was influenced by the explant. Seven out of the eleven (63.63%) cassava genotypes studied showed capability to undergo somatic embryogenesis.

Key words: Cassava, picloram, somatic embryogenesis.

INTRODUCTION

Cassava *(Manihot esculenta* Crantz) is the fourth most important source of calories in tropical and sub-tropical areas. On dry weight basis, its tuberous roots contain up to 85% starch (Cock, 1985), and the roots provide food for over 500 million people in developing countries (Roca et al., 1992). Because of its hardiness and tolerance to adverse environmental conditions such as infertile acid soils, cassava is often grown as a subsistence crop on marginal soils.

Nutritionally, the low protein content of cassava is a problem for people whose diet is based largely on this plant. The values below are the nutrient composition of fresh cassava root (per 100 g of edible portion); carbohydrate (34.7 g), protein (1.2 g), fat (0.30 g),

calcium (33 mg), and vitamin C (36 mg) (Oyewole and Asagbra 2003). The peels are richer in protein, fat and ash than the edible portions (Hahn et al., 1992). Cassava constitutes a greater part of the diets of most Africans south of the Sahara, and the demand for the crop has been on a steady increase. Although traditional breeding programs in cassava have been successful in the development of improved cultivars (Hershey and Jennings, 1992), the high degree of heterozygosity, irregular flowering in some cultivars, low seed set, and variable germination rates are limitations to fast progress via classical plant breeding.

Genetic engineering of cassava, using somatic embryos can complement traditional breeding programs in several areas such as the development of disease resistant varieties (Fauquet et al., 1992), modification of starch quality (Salehuzzaman et al., 1993), reduction in the level of cyanogenic glucosides (Koch et al., 1994) and extension in the shelf life of the harvested tubers (Thro et al., 1996). In addition to its use in breeding, rege-

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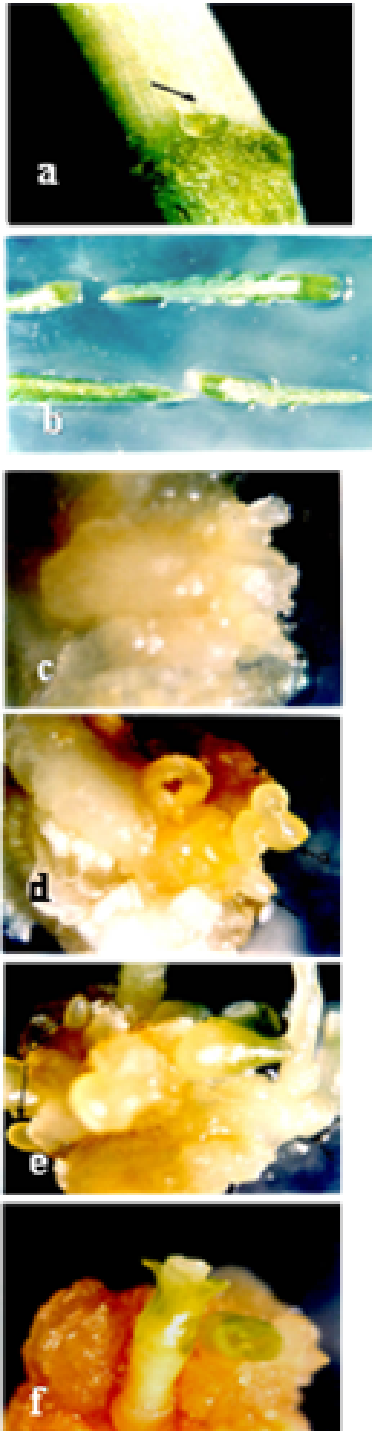*Corresponding authors E-mail: jatehnkeng@cgiar.org

Figure 1. a. Axillary meristem (at the tip of arrow) on six weeks old *in vitro* cassava (*M. esculenta* Crantz) before excision for culturing. **b**. Immature leaf lobes of six weeks old cassava cultured with the abaxial surface in contact with the culture medium. **c**. Cassava explant showing heart shaped embryo at the advanced stage of development and hard brown callus in the induction medium. **d**. Cassava explant showing, early stage torpedo, late stage torpedo shaped embryos, and friable embryogenic callus in the induction medium. **e**. An explant of cassava showing embryo at the cotyledonary stage, torpedo shaped embryos and friable embryogenic callus in the induction medium. **f**. Germination of horn shaped somatic embryo of cassava in the maturation medium.

neration to plantlets through somatic embryos offer a unique approach to overcoming the problem of shortage of improved planting materials in cassava. This technique requires less labour since somatic embryos can grow into complete plants without separate rooting and shooting steps (Parrot et al., 1991).

Somatic embryogenesis in cassava was first described by Stamp and Henshaw (1982). Somatic embryos are formed if leaves or meristems (Mathews et al., 1993) or zygotic embryos (Konan et al., 1994b) are subcultured on a medium supplemented with auxins such as picloram, 2,4-dichlorophenoxy acetic acid (2,4-D) or dicamba. Sofiari (1996) also initiated and obtained mature somatic embryos using napthaline acetic acid (NAA).

An efficient regeneration system is a *sine qua non* for successful genetic transformation aimed at improving cassava agronomic traits, resistance to viral, bacterial and fungal diseases, resistance to insect attack and high stable productivity.

A regeneration system based on embryogenesis has been developed for cassava (Stamp and Henshaw 1982, 1987a; Li et al., 1996; Mathew et al., 1993). The efficiency of this regeneration system is however, limited to a few cultivars (Stamp and Henshaw, 1982). Development of a regeneration system that is genotype-independent will widen the range of cassava genotypes used in transformation. Given the importance of somatic embryos in the production of planting materials and genetic transformation, this study has as its objective the screening of selected African cassava germplasm for their ability to undergo somatic embryogenesis.

MATERIALS AND METHODS

Plant materials

The experiment was carried out in the Tissue Culture Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. *In vitro* derived plantlets, of eleven cassava genotypes were used for this experiment, These consisted of TME 8, TME 594, TME 596, SL80/40, I60142, I95/0528, I96/0016, I96/0035, I96/0860, I96/1439 and Z95/0826.

Induction and maturation of somatic embryos

Bulb-like axillary meristem (Figure 1a) and immature leaf lobes (Figure 1b) measuring about 1 to 6 mm obtained from about six weeks old *in vitro* derived plants were used. Immature leaf lobes of this size have greater capabilities for somatic embryos induction (Raemakers et al., 1993). These explants were cultured on Murashige and Skoog (MS) media (1962), supplemented with 20 g/l sugar, 2 µM $CuSO_4$ and 0.6% agar. Picloram was used at 8 and 12 mg/l. The pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15 min. The media were poured into 9 cm diameter petri dishes under a sterile laminar flow hood; 10 ml of the medium were poured per petri dish and allowed to cool and solidify.

The axillary meristems were extracted with the aid of a dissecting microscope, using a sterile forcep, syringe and needle. The forcep was used in holding the tender stem in place and needle for carefully removing the overlapping primordial leaves to expose the axillary meristem. The excised meristems were placed on media

containing 8 and 12 mg/l picloram and the petri-dishes sealed with parafilm. The immature leaf lobes were also exposed and excised and placed on media containing the two levels of picloram. The abaxial side and midrib of the explants were placed in contact with the medium. The experiment was a three-factor factorial in which the factors were genotype, explant and picloram concentration. Experimental design was completely randomised design (CRD) with six replicates.

Ten pieces each of axillary meristem and leaf lobe were plated per petri dish containing either 8 or 12 mg/l picloram. The petri dishes were incubated in the dark at 28 ± 2°C for 30 days. After 30 days, each petri dish was microscopically examined to determine the number of explants forming pro-embryos. This was expressed as a percentage of the number of exlpants in the petri dish. The structures were transferred to fresh medium with the same composition. Two weeks later, number of globular, heart shaped and torpedo-shaped embryos were assessed. Individual explants were scored for each of the somatic embryo developmental stages, since it is possible to have all the three stages of development (globular - heart, and torpedo shaped) on a single explant.

Maturation of somatic embryos

Explants that had majority of their embryos at the torpedo stage were transferred to an MS-based maturation medium supplemented with vitamins, 20 g/l sucrose, 0.1 g/l benzyl amino purine (BAP), 2 µM $CuSO_4$, and 0.6% agar. The pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15 min. The cultures were kept under a 12-h photoperiod. After seven days the mature embryos were observed for the cotyledonary stage, characterized by the greenish horn shape outgrowth with signs of germination (Figure 1f).

RESULTS

Proembryo formation

There were significant differences ($p < 0.01$) among the cassava genotypes for embryo formation. Averaged over explants and picloram concentrations, proembryo formation ranged between 0.0% for SL80/40 and 33.6% for TME 596. On the basis of percentage proembryo formation, the genotypes were grouped into four viz; very poor, with less than 10% proembryos (SL80/40 and I60142), poor with 10 to 20% proembryos (Z95/0826, I96/0528, I96/0860, TME 8 and I96/1439), fair with a proembryo formation rate of 20 to 30% (I96/0035 and I96/0016) and good with greater than 30% proembryo formation (TME 596 and TME 594). Differences due to explant were also significant ($\rho < 0.01$). In general, embryo formation was better with leaf lobes except in 96/0016 (Table 1). Proembryos were not formed with both explants in SL80/40. Percentage of proembryo formation with leaf lobes was 55% higher than that of axillary meristem. Differences due to the picloram concentration were, however, not significant. There was a significant genotype x explant interaction, indicating that the differences among the cassava genotypes for proembryo formation were influenced by the explant. This was most pronounced in TME 8 and TME 594 for which proembryo formation with leaf lobe relative to axillary meristem was higher by 23.6%, and 18.0% and respec-

tively. Experimental coefficient of variability was high (61.3%), suggesting a low level of precision for treatment results and the likelihood that other yet-to-be identified factors play a role in proembryo formation (Table 2)

Developmental stages during somatic embryogenesis

Many of the genotypes that formed organized embryogenic structures did not progress beyond the globular stage of the somatic embryo development. Only seven out of the eleven genotypes went through all the somatic embryo developmental stages (globular, heart-shape, and torpedo; Figure1c - f). These included all the genotypes grouped on basis of proembryo formation as good (TME 596 and TME 594), fair (I96/0035 and I96/0016), and poor (I96/0860, TME 8 and Z95/0826). The response was strongly influenced by genotype. Genotype accounted for 43.25% of the total sum square. Seven genotypes; TME 596, TME 594, I96/0016, I96/0860, TME 8, Z95/0826 and I96/0035, went through the somatic embryo developmental stages (globular, heartshape, torpedo and cotyledonary stages). From the 51 explants that formed proembryos, 27 developed to the cotyledonary stage.

For TME 596, five of the seven leaf lobes (71.4%) in the 12 mg/l picloram medium that developed embryos structures eventually reached the torpedo stage. Only two out of four (50%) of the axillary meristems in the 12 mg/l picloram medium that developed embryo structures reached the torpedo stage. In the 8 mg/l picloram medium, three out of the five (60%) embryo-forming axillary meristems developed to the torpedo stage while two out of five (40%) developed to the torpedo stage for leaf lobes. On average it took 56 days from the day of induction to the torpedo stage. For TME 594, only two out of five (40%) embryo forming leaf lobes in the 8 mg/l picloram medium developed to the torpedo stage. For this genotype a large number of embryos from both the axillary meristems and leaf lobes in the 8 mg/l and 12 mg/l picloram media died after initiation. It took 58 days to reach the torpedo stage.

One leaf lobe out of four (25%), and one axillary meristem out of three (33%) for Z95/0826 in the 8 mg/l picloram medium developed to the torpedo stage, while three out of six (50%) leaf lobes in the 12 mg/l picloram medium developed to the torpedo stage. It took 55 days from the day of induction to the torpedo stage. In genotype I96/0016, three out of five (60%) leaf lobes that formed embryo structures in the 12 mg/l picloram medium developed to the torpedo stage. Many embryos were also lost in this genotype, especially from the leaf lobes cultured in the 8 mg/l picloram medium. None survived among the axillary meristems cultured in the 12 mg/l picloram medium. For this genotype, it took 65 days from the day of culture to torpedo stage.

For genotype I96/0860, embryos that developed to tor-

Table 1. Mean proembryo formation of leaf lobes and axillary meristems of eleven cassava genotypes.

Genotypes	Explant	Proembryo formation (%)	SD
TME 596	Axillary meristem	31.3	6.5
	Leaf lobe	35.8	10.5
TME 594	Axillary meristem	25.4	17.4
	Leaf lobe	43.4	11.4
I96/0016	Axillary meristem	27.2	6.9
	Leaf lobe	31.1	15.8
I96/0035	Axillary meristem	27.3	6.6
	Leaf lobe	22.2	8.4
I96/1439	Axillary meristem	16.7	8.4
	Leaf lobe	21.2	13.5
TME 8	Axillary meristem	5.3	3.2
	Leaf lobe	28.9	11.7
I96/0860	Axillary meristem	12.1	7.3
	Leaf lobe	17.8	12.4
I96/0528	Axillary meristem	11.3	9.1
	Leaf lobe	20.4	11.4
Z95/0826	Axillary meristem	10.3	8.1
	Leaf lobe	4.3	7.5
I60142	Axillary meristem	0.0	0.0
	Leaf lobe	3.1	1.2
SL80/40	Axillary meristem	0.0	0.0
	Leaf lobe	0.0	0.0

pedo were obtained only from two leaf lobes cultured in the 12 mg/l picloram. It took 59 days from the day of induction to the torpedo stage. For TME 8, two out of six leaf-lobe (33.3%) explants cultured in the 12 mg/l picloram medium developed to torpedo stage. Topedo shaped embryo was recovered from only one of the five explants (20%) of I96/0035. This was a leaf lobe cultured in the media containing 12 mg/l picloram. Development in the 8 mg/l picloram medium did not proceed beyond proembryo formation.

Performance in maturation medium

Of the seven genotypes whose explants had torpedo stage embryos and that were moved to the maturation medium, five developed further to the cotyledonary stage and germinated (Figure 1f). For TME 596, torpedo embryo-shaped embryos on three out of the five leaf-lobe explants in the 8 mg/l picloram medium, and four out of five leaf-lobe explants in 12 mg/l picloram medium germinated. Torpedo shaped embryos from the four axillary meristems at the 8 mg/l picloram germinated while embryo germination occurred on one out of the two from the 12 mg/l picloram medium. For Z95/0826, germination was observed on the only axillary meristem with torpedo shaped embryo. Similarly, embryos from the

only leaf lobe in the 8 mg/l picloram medium germinated. Embryos on two out of the three leaf-lobes explant in the 12 mg/l picloram medium germinated. Embryos from all the leaf lobe of TME 8, I96/0035 and I96/0860 in the 12mg/l picloram medium germinated. None of the embryos from the leaf lobes of I96/0016 and TME 596 in 12 mg/l germinated in the maturation medium. The most outstanding genotype was TME 596, which formed embryos that developed to the cotyledonary stage from the two explants and from the two picloram concentrations while the worst performance was shown by SL80/40 which did not develop any proembryo.

DISCUSSION

Genetic improvement of cassava by transformation requires the development of a regeneration system to be of wide applicability. Such regeneration system should be genotype-independent. In this study, seven of the eleven genotypes produced somatic embryos from either or both axillary and immature leaf lobes in media containing picloram as the source of auxin. Picloram is one of the best auxins for inducing somatic embryogenesis in African cultivars of cassava (Taylor et al., 1993). The two picloram concentrations gave similar results; the lower rate of 8 mg/l is therefore considered more eco-

Table 2. Analysis of Variance of the effect of genotype, explant and picloram concentration on somatic embryo development in the induction medium.

Source of Variation	DF	Sum of squares	Mean squares	F Value
Replicate	5	2358.5	471.7	3.70**
Explant	1	2805.5	2805.5	21.9**
Genotype	10	31628.1	3162.8	24.7**
Picloram	1	29.5	29.46	0.2 [NS]
Explant x Picloram conc	1	73.3	73.3	0.6 [NS]
Genotype x Picloram conc	10	831.4	83.1	0.7 [NS]
Genotype x Explant	10	3840.7	384.0	3.0**
Genotype x Picloram conc x Explant	10	4134.20	413.4	3.2**
Error	215	27427.1	127.6	
Total	263	73128.4		
CV (%)	61.3			

** = Significant at 0.01 probability level.
NS = Not significant.

nomical. Previous studies (Ng, 1992; Raemakers, 1993; Ng and Adeniyi, 1994) had indicated 12 mg/l picloram as more favorable for inducing somatic embryos in some African cassava cultivars. It may be worthwhile to investigate the effects of picloram concentrations higher than 12 mg/l on genotypes that respond poorly to somatic embryo induction. In this study, four categories of tissues were observed viz. organized embryogenic structures (OES), non-embryogenic callus (NEC), hard brown callus (HBC) and friable embryogenic callus (FEC). The type of tissues developed was, to a great extent, genotype dependent. For example, organized embryogenic tissue was common to TME 596, TME 594, I96/0860, I96/0016 and TME 8 and non-embryogenic callus to I96/1439, and I96/0035. The hard brown callus was common to all genotypes especially in the axillary meristem cultured in the 8 mg/l picloram medium, while friable embryogenic callus was characteristic of those that formed embryos. Nigel et al. (1996) also observed the four types of tissues in a study involving cassava genotypes M col. 1505 and TMS 60444 at varying concentrations of picloram. Vassil (1995) reported that the friable embryogenic tissue disperse easily and proliferate in liquid cultures to produce high quality embryogenic suspension cultures in which large majority of cells are totipotent. He also identified this tissue as an ideal target tissue for use with direct gene transfer systems because they maximize the probability of the insertion and integration of foreign genetic material into large numbers of morphologically competent cells.

The genotypes that responded to the picloram medium treatment in the induction process passed through the globular to the cotyledonary stages, though many of the induced embryos that remained in the globular stage were overgrown by callus. The loss of embryogenic structures prior to formation of embryos was a common occurrence in this study, especially in TME 594, and I96/0016. Konan et al. (1994a) had reported this occurrence and further observed that it yielded some other adventitious organs, like "leafy", roots and occasionally shoots. The loss of embryogenic competence may be due to the cytokinin BA (0.1 mg/l) used in the maturation medium. Kysely and Jacobsen (1990) previously reported the inhibitory effect of BA on embryo development.

The emergence of proembryo in the induction media was observed within 27 to 35 days after subculture for the genotypes that responded. This is relatively long compared to the 15 to 25 days observed by Konan et al. (1994a). These differences may not be unconnected with differences between the cassava genotypes used in this study since regeneration in cassava is largely recognized as a function of genotype and culture conditions (Geobel-Tourant et al., 1993).

In this study leaf lobes demonstrated higher success for proembryo formation with an average of 21.7% compared to axillary meristem with an average of 13.8%. Using this regeneration method transplantable shoots and genetic transformation material can be regenerated from leaf lobes within 55 to 70 days.

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