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Full Length Research Paper

Optimization of somatic embryogenesis and selection regimes for particle bombardment of friable embryogenic callus and somatic cotyledons of cassava (*Manihot esculenta* Crantz)

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Although a number of transformation systems and selection regimes have been developed for cassava (*Manihot esculenta* Crantz), they have only been applied in a limited number of genotypes. This limitation of the applicability of the systems is due largely to variation in morphological responses of different genotypes to the regeneration and transformation procedures employed, which underscores the need to study all possible parameters for any given cultivar. Using two cultivars ("Rosinha" and "Bujá Preta") we made an attempt to improve the frequency of somatic embryogenesis (SE), establish friable embryogenic callus (FEC) lines from developing somatic embryos and evaluate the effect of different concentrations of the antibiotics kanamycin, on secondary somatic embryogenesis (SSE) and of paromomycin, on FEC in proliferation and histodifferentiation media. Further, we report on transient expression of the visual marker gene *uidA* following particle bombardment of FEC from 'Bujá Preta' and of cut pieces of green somatic cotyledons from 'Rosinha', using the plasmid pBI426. Higher number of embryos, which emerged as early as 8 days after culture on MS medium (CIM), was obtained when abaxial part of the explant was in direct contact with the medium as against the adaxial side, and this did not depend on the explant size (0.5 cm² or 2.5 cm²). Highly friable and embryogenic callus was obtained on GD medium supplemented with 2% (w/v) sucrose and 12 mg/L picloram. While paromomycin, at concentration of 60 mg/L arrested the proliferation and histodifferentiation of FEC, kanamycin in CIM containing explants undergoing SSE led to a decrease in their embryogenic potential resulting in tissue death at 50 mg/L. The highest transient expression of *uidA* gene in FEC was observed combining plasmolysis, M5 particle and a helium pressure of 1,200 psi, while in somatic cotyledons, the highest expression was observed when M5 particle was used along with the helium gas pressure of 900 psi.

Key words: FEC, *Manihot esculenta,* somatic embryogenesis, selective agent, transient expression.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the third largest tropical crop used as a staple food, particularly in Africa, Asia and South America (Stupak et al., 2006). An estimated 700 million people obtain more than 500 Kcal per day from the crop and more than 500 million people consume 100 Kcal per day from cassava (Taylor et al*.,* 2004). Globally, dependence on the crop is expected to increase with a projected 60% boost in production by 2020 (Aerni, 2006). While conventional methods of breeding have failed to fully address the problems associated with the production and utilization of the crop (Puonti-Kaerlas, 2000), transgenic technologies represent an alternative tool that can complement traditional breeding to extend its pool for useful gene sources over

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Abbreviations: CIM, Cassava somatic embryogenesis induction medium; **CMM**, Cassava somatic embryogenesis maturation medium; **FEC**, friable embryogenic callus; **GD2P50**, GD medium + 2% sucrose and 12 mg/L picloram; **MS2NAA5**, MS medium+ 2% sucrose and 1mg/L NAA; **SE**, somatic embryogenesis; **SSE**, secondary somatic embryogenesis.

species barriers while offering the possibilities of transferring single trait without encountering the problems often associated with traditional breeding (Taylor et al., 2004).

An important prerequisite for the development of genetic transformation system is the availability of morphogenic culture that can be used in gene transfer techniques (Taylor et al., 1996, 2004). In cassava, this can be achieved through somatic embryogenesis. First reported by Stamp and Henshaw (1982) from zygotic cotyledons and then from clonal leaf materials of cassava, the general strategy is to induce the formation of somatic embryos from explants on a Murashige and Skoog (Murashige and Skoog, 1962) (MS) based medium supplemented with auxin, from which embryos can be used to regenerate plants that are clones of the parent material while preserving all traits of the original germplasm. However, despite the apparent success in the production of somatic embryos in cassava, their use as targets in most regeneration and genetic transformation systems has not been quite encouraging (Raemakers et al., 1997; Zhang and Puonti-Kaerlas, 2000; Sarria et al., 2000; Siritunga and Sayre, 2003). This is because both primary and secondary somatic embryos seem to have a multi-cellular origin and this may lead to the production of chimeras, thereby increasing the chances of somaclonal variation (Stamp and Henshaw, 1987; Raemakers et al., 1997). On the other hand, the system of somatic embryogenesis that produces FEC is preferred due to the fact that the progenitor cells of these structures are located on the surfaces of preexisting FEC units, thus emerging cells are easily accessible to all techniques of gene transfer (Taylor et al., 1996). FEC therefore, represents the initial stage of embryogenic development, as such is advantegeous in the transformation of plants because an individul cell has the chance of behaving independent of its neighbouring cell to form a new embryogenic unit.

We have earlier established protocols for the induction of SE in ten cassava genotypes (Machado et al., 2007). In this work, we report on the improvement of the frequency of SE and embryo conversion of cut pieces of somatic cotyledons, establishment of FEC lines from somatic embryos as well as evaluation of the effect of different concentrations of the antibiotics kanamycin, on secondary somatic embryogenesis (SSE) and of paromomycin, on FEC in proliferation and histodifferentiation medium using two genotypes (‘Rosinha’ and ‘Bujá Preta’, respectively). In addition, we report on transient expression of the visual marker gene *uidA* following particle bombardment. Our findings shall form the basis for effective transformation of cassava with other reporter genes and subsequently with genes of agronomic importance.

MATERIALS AND METHODS

Plant materials

The cassava varieties used in this study (“Rosinha” and “Bujá Preta”) were obtained from the cassava germplasm bank maintained at the Agronomy School, Federal University of Ceará, Fortaleza, Brazil. For the establishment of *in vitro* cultures, the method described by Machado et al. (2007) was used. Fresh stem cuttings (20-30 cm) with 5-8 nodes were obtained from mature plants. The cuttings were planted in plastic trays (50 x 30 x 8 cm) filled with vermiculite and watered with half-strength MS salts and kept under green house conditions. When the newly sprouted stems reached 2 cm, they were cut from the mother plant, taken to the lab and sterilized by immersion in a solution of 1.5% sodium hypochlorite and 0.01% Triton X-100 for 5 min. Shoot tips containing up to three leaf primordia were aseptically dissected and transferred to test tubes (15 x 2.5 cm) containing 10 ml of solidified MS medium supplemented with 0.1 mg/L benzyl adenine (BA). The tubes containing the explants were incubated at 26 ± 2ºC in a growth chamber programmed to provide a light and dark cycle of 16/8 h provided by white fluorescent lamps (25.3 µmol m^{-2} s^{-1}). The cultures were maintained by monthly subculture of nodal explants in the same medium. For all media used, pH was adjusted to 5.8 with 1.0 N NaOH and agar (0.5% for all GD medium and 0.8% for all MS medium), was added before autoclaving.

Cyclic somatic embryogenesis

Primary somatic embryogenesis was induced by culturing isolated shoot apex obtained from *in vitro* plants, in Petri-dish containing cassava induction medium (CIM) which is composed of MS medium supplemented 8 mg/L picloram. Maturation of embryos was achieved by transferring the primary embryos into cassava maturation medium (CMM) containing MS medium supplemented with 0.1 mg/L BAP. A cyclic system of secondary somatic embryogenesis was established by subjecting cut pieces of cotyledons from mature somatic embryos to CIM. The effects of the position of the explant (abaxial and adaxial) in relation to the medium and of cotyledon size (0.5 and 2.5 cm²), on the frequency of SE were evaluated.

Induction of FEC and embryogenic suspension

FEC was induced by culturing developing somatic embryos on Gresshoff and Doy (1974) (GD) medium supplemented with 2% sucrose (w/v) and 12 m/L picloram (GD2P50) in a 16 h photoperiod. The emergence of FEC and FEC production capacity of each explant was monitored over a period of 84 days. Embryogenic suspension was prepared by culturing FEC in a liquid in flask and kept on a gyratory shaker (MARCONI-MA 140 CFTT) at 120 rpm in 16 h photoperiod. To regenerate embryos from FEC, the tissues were cultured on MS medium containing 2% (w/v) sucrose and 1 mg/L NAA (MS2NAA5) and kept in 16 h photoperiod.

Determination of selection pressure

Cut pieces of green cotyledons originating from CMM were cultured in the presence of kanamycin at the concentrations of 0.0, 0.05, 0.5, 1.0, 2.5, 5.0 and 50 mg/L in CIM and with 0.0, 0.5, 1.0, 2.5, 5.0, 10, 25 and 75 mg/L in CMM. For each treatment, 3 replicates were made with each replicate represented by 1 plate containing 10 explants per plate giving a total of 30 explants per treatment.

Hundred milligrams (100 mg) of FEC was cultured in GD2P50 supplemented with filter sterilized paromomycin in the following concentrations: 0.0, 5.0, 10.0, 45.0, 50.0 and 60.0 mg/L. FEC fresh weight and the appearance of undesirable callus as well as the death of individual explants were recorded 30 days later. The same amount of FEC was cultured on MS2NAA5 supplemented with filter sterilized paromomycin in the concentrations of 0.0, 5.0, 10.0, 40.0, 50.0 and 60.0 mg/L. The emergence or otherwise of embryos was

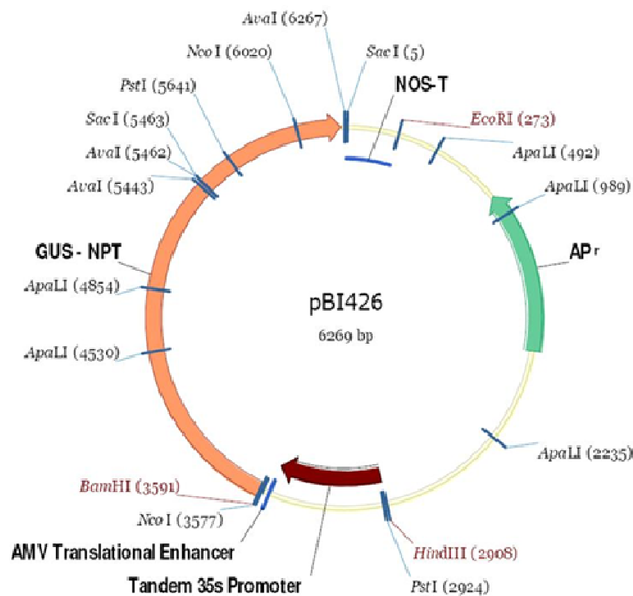

Figure 1. Restriction map of the plasmid pBI426 (35S-35S, double 35S CaMV promotor from *Cauliflower mosaic virus*; AMV, leader sequence from *Alfalfa mosaic virus*, *uidA*-*npt*II, fused genes coding for β-glucoronidase (GUS) and neomycin phosphotransferase (nptII); NOS-T terminator from nopaline synthase.

observed after two weeks. Each treatment consisted of 4 clusters of 100 mg FEC (16 clusters per treatment) replicated three times.

Viability test

Paromomycin-treated FEC was used to carry out viability test using fluorescein diacetate (FDA) by adopting the method described by Schöpke et al. (1996) with slight modifications. An aliquot of 40 µL FEC from each antibiotic treatment was stained with 0.05% FDA in liquid GD2P50 for ten minutes and then mounted on microscopic slides. All the slides were viewed with optical microscope (Olympus System Microscope BX 60), under white light and fluorescent (Olympus System Attachment Model BX-FLA, Olympus). Pictures were taken with inbuilt camera (Olympus Photomicrosgraphics System PM 20). For each treatment, three slides were prepared and the experiment repeated 3 times.

Transformation

A particle delivery system developed by EMBRAPA/CENARGEN (Rech et al., 2008) which was based on the Sanford and Davit (1991) model was used to bombard green cotyledons of "Rosinha" and FEC from "Bujá Preta". The distance between the retention plate and the target tissue was 80 mm and the RH of the laboratory was 44 ± 4%. The vector used was pBI426 (Figure 1); which contains the genes *uidA* and *nptII* (Dalta et al., 1999) fused under the control of 35S promoter from *Cauliflower mosaic virus* (CaMV) bearing a leading enhancer sequence from *Alfalfa mosaic virus* (AMV). Sixty milligram each, of two different tungsten particles; M5 and M10 (Sylvania) (corresponding to 0.5 and 1.0 µm average diameters, respectively) were transferred to centrifuge tubes containing 1 mL of 70% ethanol and centrifuged for 15 min. The particles were re-suspended in 50% glycerol. Fifty microliters of the particle was mixed with 5 µL of plasmid DNA (1 ug/µL), 50 µL of 2.5M $CaCl_2$ and 20 µL of 0.1 M spermidine. The mixture was shaken gently for 10 min and an aliquot of 3.2 µL of this was used per bombardment.

For particle bombardment of FEC, cell suspension of the tissue was prepared at least 10 days before. Fractions ranging in size between 100-500 µm were obtained from the suspension by first vortexing the FEC for 15 s and passage through sieve (40 - 60 mesh). For each bombardment, approximately 250 mg of the tissue was spread on the bombardment plate (5 cm) as a monolayer. The effect of particle size and pre-treatment of the tissues with 0.2 M equimolar concentrations of sorbitol and mannitol 4 h before and 16 h after bombardment of the FEC, on transient expression of *uidA* was evaluated after histochemical analysis using the method of Jefferson (1987).

For the particle bombardment of the somatic embryos, cut pieces of cotyledons (0.5 cm^2) were placed in bombardment plates. The effects of gas pressures (900 and 1,200 psi) and particle size (M5 and M10) on the transient expression of *uidA* were studied. Where necessary, data obtained was analyzed using SAS (SAS Institute, 1992-1998).

RESULTS AND DISCUSSION

Effect of the position of explants and explant size on SSE

The induction of SSE was influenced by the position of the explants in relation to the culture medium. Although no significant difference was observed in terms of the frequency of SSE, there was a tendency of obtaining higher number of embryos when the abaxial part of the explant was in direct contact with the culture medium as against the adaxial side (Table 1). In addition, more callus formation was observed in the adaxial treatment. As early as 8 days after culture, the explants under the abaxial treatment showed evidence of embryogenesis and by the 12th day, globular stage embryos could be clearly observed. When present, callus was observed only around the region of cut. Although Raemakers et al. (1993) observed that embryos emerge from the adaxial part of the explants, this is the first time such parameter is studied and has been found to influence SSE in cassava.

Since embryos seem to emerge from the cells around the adaxial side of the explants (Stamp, 1987), we expected a kind of correlation in the frequency of SSE and the number of embryos produced per explants on the one hand, and the explants size on the other. However, the size of explant did not seem to affect, neither the frequency of SSE nor the quantity of embryos produced (Table 1). We observed that even when explant size was increased from 0.5 to 2.5 cm^2, no significant difference existed in the frequency of SSE. Most researchers actually use an explant of the size 0.5 cm (Zhang and Puonti-Kaerlas, 2000; Zhang et al., 2000), irrespective of the regeneration and transformation systems used. This finding not withstanding, the position of the explant should be considered as an important factor in the attempt to improve the frequency of SE in any cultivar of cassava.

Table 1. Effect of position and size of explant on the induction of somatic embryogenesis in cassava cv. Rosinha.

Treatments	Frequency of SSE (%)	Mean number of embryos ± SE
Abaxial (0.5 cm²)	100 [a]	64.67 ± 10.33 [a]
Adaxial (0.5 cm²)	98.3 [a]	42.83 ± 23.80 [b]
Abaxial (2.5 cm²)	100 [a]	60.88 ± 16.93 [a]
Adaxial (2.5 cm²)	94.7 [a]	41.05 ± 24.10 [b]

Values with the same letters along the same column indicate that there is no significant difference in the treatments (Tukey 5%).

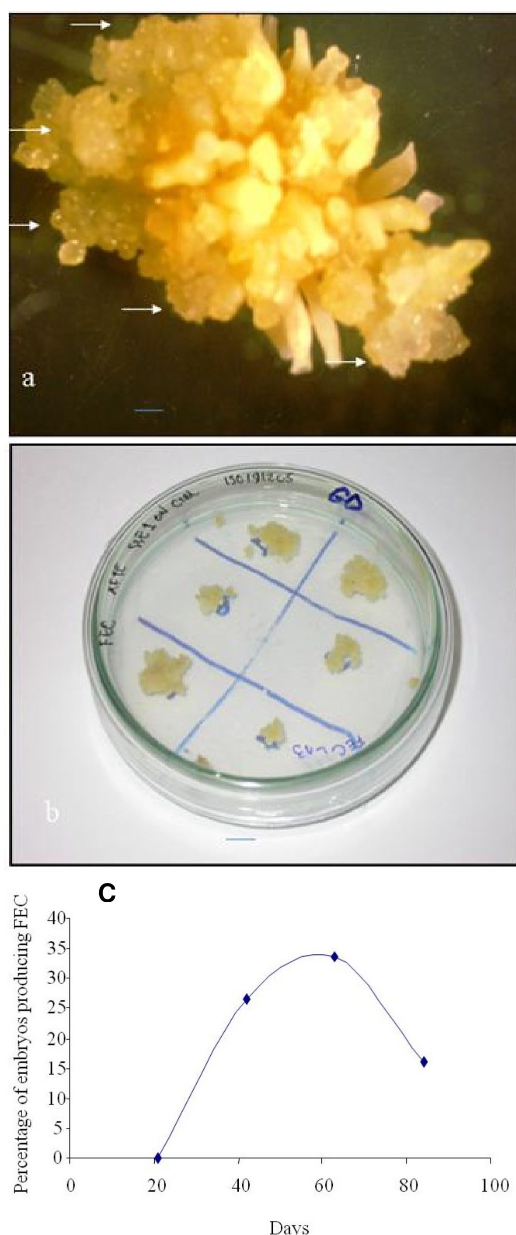

Figure 2 - Emergence of FEC from somatic embryos in cassava (*Manihot esculenta*) cv Buja Preta, cultured in GD2P50 (a) and their isolotion on the same medium (b). The FEC production capacity of the fragments is highest at the end of the second cycle of 21 days sub-culture (c) . Bars and arrows in a and b represent 1 cm.

Induction and maintenance of FEC

Fragments of somatic embryos started producing FEC about 21 days after culture in GD2P50 (Figure 2a). Over 30% of the individual explants produced FEC within two cycles of 21 days reaching the highest peak by the second subculture (Figure 2c). By the third cycle, there was a fall in the production of callus and most of the explants have either completely converted to FEC or died altogether. The FEC produced gave a homogenous culture which was highly friable and embryogenic.

Effect of paromomycin on the proliferation and histodifferentiation of FEC

Killing curve of FEC showed that 100 mg of the tissue increased in fresh weight in GD2P50 supplemented with up to 45 mg/L paromomycin for 30 days. At concentrations higher than 50 mg/L, there was interruption in proliferation and loss of friability of the FEC (Figure 3a). Paromomycin at the concentration of 60 mg/L promptly killed the cells and caused their disintegration. This is true for both proliferation and histodifferentiation (Figure 3c). This was confirmed by a viability test (Figure 3b). At low concentrations of paromomycin (typically 5 mg/L), there was a dramatic growth of non embryogenic callus which frequently weighed less than FEC. It is possible that paromomycin, at low concentrations interferes with the hormonal balance of the cells as has been observed else where (Lin et al., 1995) thus leading to the production of callus of different morphology.

Different workers reported the use of paromomycin as a selective agent (Schöpke et al., 1996; Gonzalez et al., 1998; Taylor et al., 2001). In the case of "Bujá Preta", FEC can be selected using the antibiotic at the concentration range of 50 to 60 mg/L during proliferation and histodifferentiation. Although in some works (Puonti-Kaerlas, 2001), paromomycin has been reported to reduce the regeneration potential of some transgenic materials, it has been used with remarkable success (Schöpke et al., 1996). Reasons such as this are often given in defense of the selection systems that combine chemical and visual selective agents like luciferase (Raemakers et al., 1996). Although the use of luciferase

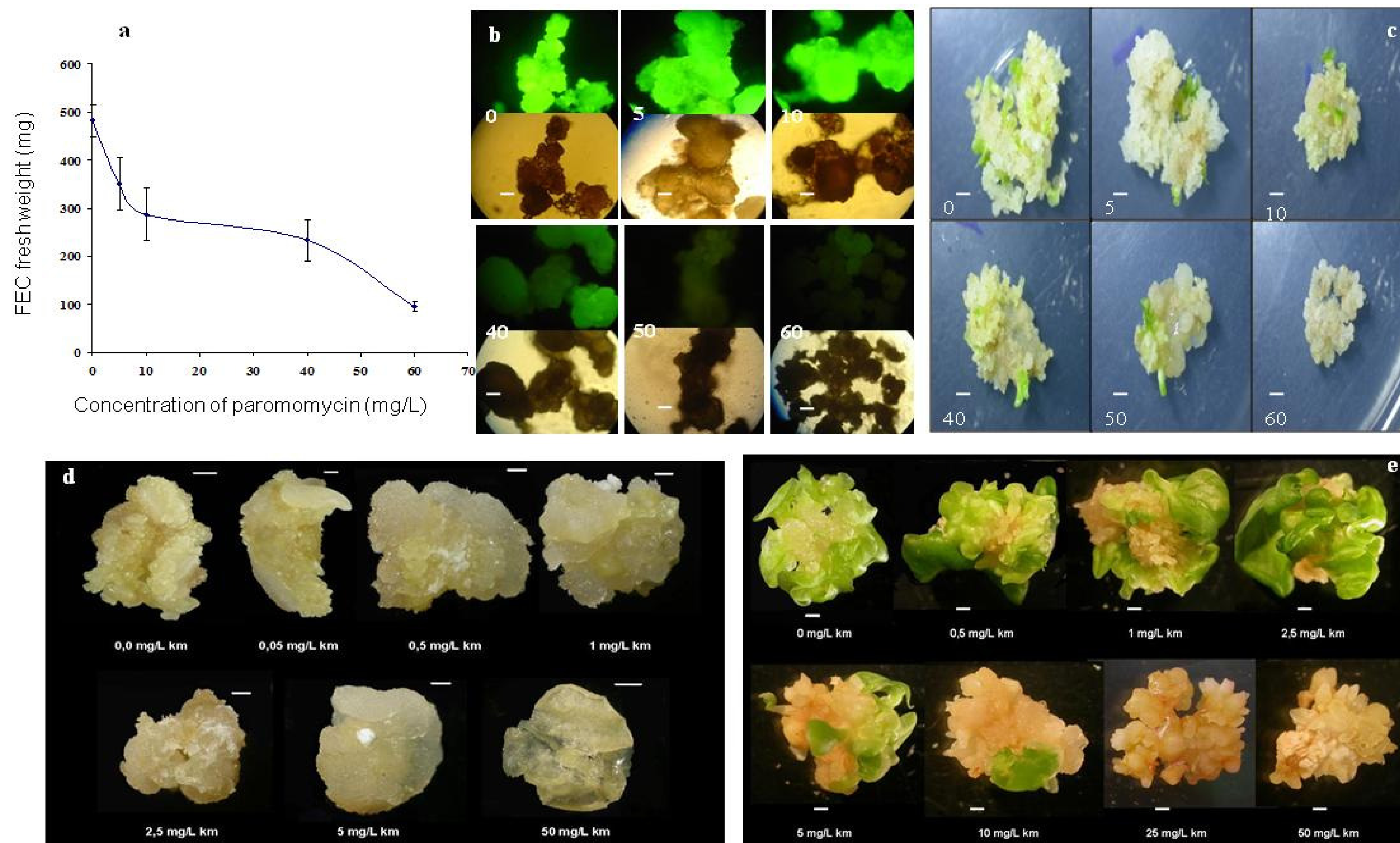

Figure 3. Effect of different concentrations of paromomycin on the proliferation and histodifferentiation of FEC from cassava (*Manihot esculenta*) cv Buja Preta (a-c) and of kanamycin on somatic embryogenesis of cassava (cv Rosinha) (d-e). Increase in fresh weight and histodifferentiation of FEC were inhibited by 60 mg/L of paromomycin (a). Viability test confirmed the death of the cells between 40 to 60 mg/L (b). Increase in the concentration of kanamycin inhibited the embryogenic capacity of the explants with accompanying callus formation (d) and maturation of embryos generated led to tissue death at 50 mg/L of kanamycin. Bars in b and c represent 0.05 and 0.5cm respectively and bars in d and e represent 0.1cm.

Table 2. Effect of the concentration of kanamycin on secondary somatic embryogenesis of cassava cv. Rosinha.

CIM + Kanamycin	Frequency of SSE (%)	Mean Number of Embryos ± SE
0.0 mg/L	96.7 [ab]	51.67 ± 21.98[ab]
0.05 mg/L	96.7 [ab]	61.67 ± 19.67[a]
0.5 mg/L	100 [a]	40.67 ± 23.33 [b]
1.0 mg/L	80 [b]	9.33 ± 8.68[c]
2.5 mg/L	20 [c]	2.00 ± 4.07 [c]
5.0 mg/L	6.7 [cd]	0.67 ± 2.54[c]
50 mg/L	0 [d]	0 ± 0.0[c]

Values with the same letters along the same column indicate that there is no significant difference in the treatments (Tukey 5%).

Table 3. Influence of helium gas pressure and particle size on the transient expression of *uidA* in cotyledons of somatic embryos from cassava cv. Rosinha.

Treatments	Frequency of GUS positive explants (%)	Maximum number of blue spots per explant	Mean number of blue spots per explant ± SE
900 psi/ M-5	98.5 [a]	122	23.15 ± 23,47 [a]
900 psi/ M-10	70.6 [b]	24	3.44 ± 5.03 [c]
1,200 psi/ M-5	47.3 [c]	11	1.08 ± 1.82 [c]
1,200 psi/ M-10	81.4 [ab]	74	10.25 ± 14.86 [b]

Values with the same letters along the same column indicate that there is no significant difference in the treatments (Tukey 5%).

may allow for the development of non transgenic tissues, escapes may be eliminated by separating the steps of visual selection and chemical selection at the regeneration stage of maturation (Snepvengers et al., 1997). While the use of luciferase selection system may be beyond the reach of some laboratories for economic reasons (Puonti-Kaerlas, 2001), an alternative system that combines the visual selection with positive selection using mannose seems promising in ensuring the elimination of non transformed tissues without compromising the putative transgenic tissues (Zhang and Puonti-Kaerlas, 2000).

Effect of kanamycin on secondary somatic embryogenesis

Increase in the concentration of kanamycin in CIM containing explants undergoing SSE led to a decrease in their embryogenic potential as evidenced by the reduction in the frequency of SSE and a decline in the number of embryos produced (Table 2). This was accompanied by increase in the production of non embryogenic callus, except in the treatment containing 50 mg/L of kanamycin, where the explants died altogether (Figure 3d-e). The callus formation may be as a result of methyllation of DNA (Schmitt et al., 1997), which could have affected the genetic expression in the embryogenic cells or as a result of hormonal imbalance due to the presence of by-products of antibiotic metabolism, since they are known to mimic growth regulators (Holford and Newbury, 1992; Lin et al., 1995).

Although our results show that kanamycin at a concentration of 50 mg/L is an efficient agent of selection, studies have shown that sub-lethal concentrations of the antibiotic should be employed. For example, Zhang and Puonti-Kaerlas (2000) used lower concentrations of kanamycin before transferring the explants to medium to higher concentrations of the antibiotic and attributed the high frequency of regeneration obtained to this strategy.

Establishment of parameters for particle bombardment of FEC and somatic cotyledons

Histochemical analysis carried out 24 h after bombardment of FEC with M5 particle without plasmolysis did not show any significant expression of *uidA*. However, FEC that was pretreated with equimolar concentrations of 0.2 M sorbitol and mannitol 4 h before and 16 h after bombardment using both M5 and M10 particles and a helium pressure of 1,200 psi, expressed the gene with the highest expression found in M5 (Figure 4). Although the frequency of transformation seems to be low, the relatively higher level observed in the cells seemed promising.

In the case of cotyledons from “Rosinha”, the highest frequency of transient expression per explant was observed when M5 particle was used along with the helium gas pressure of 900 psi (Table 3). In addition, the

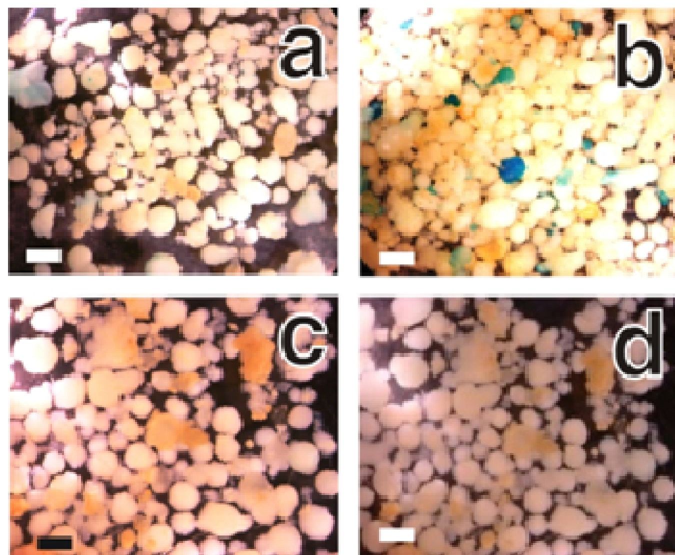

Figure 4. Transient expression of *uidA* in cassava (*Manihot esculenta*) following bombardment of FEC (cv. Buja Preta) using M5 (a and b) and M10 (c and d) tungsten microparticles of plasmolised (b and d) and non- plasmolised (a and c) explants. Bars represent 0.5 mm.

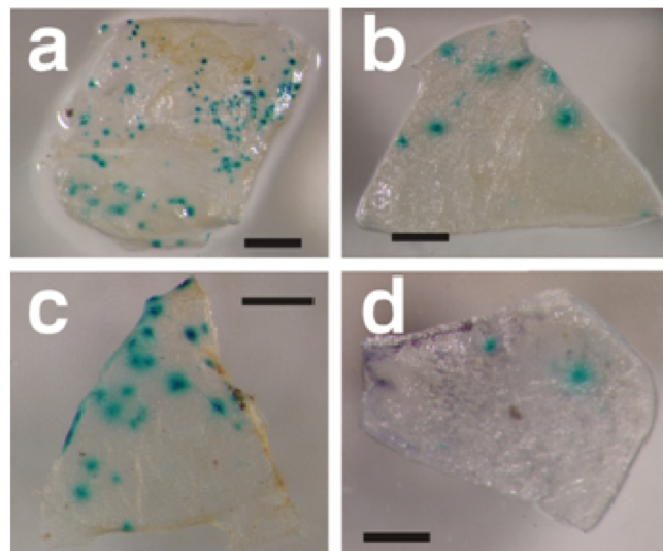

Figure 5. Transient expression of *uidA* in cassava (*Manihot esculenta*) following bombardment of cotyledons from the "Rosinha" combining a helium pressure of 900 psi (a and b) and 1,200 psi (c and d), with particle M5 (a and d) and M10 (b and c). Bars represent 0.1 cm.

explants under this treatment (900 psi+M5) presented smaller but better defined and evenly distributed blue spots than explants from other treatments (Figure 5).

The use of particles with low diameter has been reported to result in high frequency of transient expression in different plant species (Kemper et al., 1996; Yang et al., 1999; Devi and Sticklen, 2002; Tee and Maziah, 2005). This is because particles with lower diameters tend to group less during the process of DNA precipitation and when released, they separate from each other better as such penetrate less into the cells. In addition, low pressure is correlated with better distribution of particles on the bombarded material (Rasco-Gaunt et al., 1999). Therefore, the combination of these two parameters allows for the recovery of plant tissues with relatively high transient expression in cassava.

Conclusion

Our findings indicate that higher frequency of SE and higher number of explants per embryo is obtained when explant is positioned with its abaxial side in direct contact with the culture medium. We have established cultures of independent FEC lines originating from different embryogenic cycles. While up to 50 mg/L of kanamycin is required to select green cotyledon of somatic embryos of cassava (cv Rosinha), lower concentrations should suffice at initial days following transformation. When FEC tissues were subjected to solid proliferation and histo-differentiation medium in the presence of paromomycin, the phytotoxic level of the antibiotic was also found to be around 50 mg/L. Although 50-60 mg/L of paromomycin can be used to select for putative transgenic FEC of cassava (cv Bujá Preta) in both proliferation and histodifferentiation media, and a little over 5 mg/L of the antibiotic may be used for the selection of embryogenic suspension in liquid media, we recommend slightly lower concentrations within the first few days following transformation before the adoption of these values in subsequent regeneration stages. Particle bombardment of FEC showed that higher frequency of transformation is recorded when tissues were plasmolysed and the particle M5 used and a combination of M5 particle and 1,200 psi helium pressure was most efficient in the transformation of cotyledons. Our findings shall form the basis for effective transformation of cassava with reporter genes and subsequently with genes of agronomic importance.

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REFERENCES

Aerni P (2006). Mobilizing Science and Technology for Development: The Case of the Cassava Biotechnology Network (CBN). AgBio-Forum 9: 1-14

Devi P, Sticklen M (2002). Culturing shoot-tip clumps of pearl millet [*Pennisetum glaucum* (L.) R. Br.] and optimal microprojectile bom-

bardment parameters for transient expression. Euphytica 125: 45-50.

Gonzalez De-Schöpke AE, Schöpke C, Taylor NJ, Beachy RN, Fauquet CM (1998). Regeneration of transgenic plants (*Manihot esculenta* Crantz) through *Agrobacterium*-mediated transformation of embryogenic suspension cultures. Plant Cell Rep. 17: 827-831.

Gresshoff PM, Doy CH (1974). Description of a haploid cell line from *Vitis visiflora* and importance of meiotic development of anthers for haploid culture of this and other genera. Z Pflanzenphysiologie 50: 132-141.

Holford P, Newbury HJ (1992). The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. Plant Cell Rep. 11: 93-96.

Jefferson RA (1987). Assaying chimeric genes in plants; the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-404.

Kemper EL, Silva MJ, Arruda P (1996). Effect of microprojectile bombardment parameters and osmotic treatment on particle penetration and tissue damage in transiently transformed cultured immature maize (*Zea mays* L.) embryos. Plant Sci. 121: 85-93.

Lin JJ, Assadgarcia N, Kuo J (1995). Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *Agrobacterium tumefaciens* cells. Plant Sci. 109: 171-177.

Machado TF, Bastos JLP, Ponte LFA, JUCÁ TL, Campos FAP (2007). Somatic embryogenesis in cassava genotypes from the northeast of Brazil. Brazilian Arch. Biol. Technol. 50: 201-206.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497

Puonti-Kaerlas J (2001). Molecular Biology of Cassava. Hortic. Rev. 26: 85-159.

Raemakers CJJM, Amati M, Staritsky G, Jacobsen E, Visser RGF (1993). Cyclic somatic embryogenesis and plant regeneration in cassava. Ann. Bot. 71: 289-294.

Raemakers CJJM, Sofiari E, Taylor NG, Henshwa E, Jacobsen E, Visser RGF (1996). Production of transgenic cassava plants by particle bombardment using luciferase activity as a selection maker. Mole. Breed. 2: 339-349.

Raeamkers CJJM, Rodzenboom MG, Danso MK, Jacobsen E, Visser RGF (1997b). Regeneration of plants from somatic embryos and friable embryogenic callus of cassava. Afr. J. Root Tuber Crops 2: 32-36.

Rasco-Gaunt S, Riley A, Barcelo P, Lazzeri PA (1999). Analysis of particle bombardment parameters to optimize DNA delivery into wheat tissues. Plant Cell Rep. 19: 118-127.

Rech EL, Vianna GR, Aragão FJL (2008). High efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nat. Protocol. 3: 410-418.

Sanford JC, Davit MJ (1991). An improved helium - driven biolistic device.Technique 1: 3- 16

Sarria R, Torres E, Angel F, Chavarriga P (2000). Transgenic plants of cassava (*Manihot esculenta*) with resistance to basta obtained by *Agrobacerium*-mediated transformation. Plant Cell Rep. 19: 339-344.

SAS. (1992-1998). Institute Inc. *SAS* Language reference. Version 6, Cary NC: SAS Institute Inc. 1042.

Schmitt F, Oakeley J, Jost JP (1997). Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tabacum* plants. J. Biol. Chem. 272: 1534-1540

Schöpke C, Taylor NJ, Carcamo R, Konan NKK, Marmey P, Henshaw GG, Beachy RN, Fauquet C (1996). Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) from microbombarded embryogenic suspension cultures. Nat Biotechnol. 14: 731-735.

Schöpke C, Taylor NJ, Carcamo R, Beachy RN, Fauquet C (1997). Optimization of parameters for particle bombardment of embryogenic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis. Plant Cell Rep. 16: 526-530.

Siritunga, D, Sayre RT (2003). Generation of cyanogens-free transgenic cassava. Planta, 217: 367-373.

Snepvengers E, Raemakers CJJM, Kanju E, Danso K, Van Lammeren AM Jacobsen E, Visser RGF (1997). Optimization of chemical selection of transgenic friable embryogenic callus of cassava using the luciferase reporter gene system. Afr. J. Root Tuber Crops 2: 196-200.

Stamp JA, Henshaw GG (1982). Somatic embryogenesis in cassava. Zeitschrift für Pflanzenphysiologie 105: 183-187.

Stamp JA (1987). Somatic embryogenesis in cassava: the anatomy and morphology of the regeneration process. Ann. Bot. 59: 451-459.

Stamp JA, Henshaw GG (1987). Secondary somatic embryogenesis and plant regeneration in cassava. Plant Cell Tissue Organ Cult. 10: 227-233.

Stupak M, Vanderschuren H, Gruissem W, Zhang P (2006). Biotechnological approaches to cassava protein improvement. DOI 10.1016/j.tiffs.2006.06.004, Trends Food Sci. Technol. 17(12): 634-641.

Taylor NJ, Edwards M, Kiernan R, Davey CDM, Blakesley DD (1996). Development of friable embryogenic callus and embryogenic suspension culture systems in cassava. Nat. Biotechnol. 14: 726-730.

Taylor NJ, Masona MV, Carcamo R, Ho T, Schöpke C, Fauquet C (2001). Production of embryogenic tissues and regeneration of transgenic plants in cassava. Euphytica 120: 25-34.

Taylor N, Chavarrriaga P, Raemakers K, Siritunga D, Zhang P (2004). Development of transgenic technologies in cassava. Plant Mol. Biol. 56: 671-688.

Tee CS, Maziah M (2005). Optimization of biolistic bombardment parameters for Dendrobium Sonia 17 calluses using GFP and GUS as the reporter system. Plant Cell, Tissue Organ Cult. 80: 77-89.

Yang J, Lee HJ, Shin DH, OH SK, Seon JH, Paek KY, Han KH (1999). Genetic transformation of *Cymbidium* orchid by particle bombardment. Plant Cell Rep. 18: 978-984

Zhang P, Puonti-Kaerlas J (2000). PIG-mediated cassava transformation using positive and negative selection. Plant Cell Rep. 19: 1041-1048.

Zhang P, Potrykus I, Puonti-Kaerlas J (2000). efficient production of transgenic cassava using negative and positive selection. Transgenic Res. 9: 405-415.