



Development of Transgenic Cassava Cultivars from Northeastern Brazil

By

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Abstract

Following the establishment of a cyclic system of somatic embryogenesis, in excess of 20 independent friable embryogenic callus (FEC) lines originating from different embryogenic cycles were established when fragments of somatic embryos were cultured on GD medium containing 2% sucrose supplemented with 50 μ M picloram. Histodifferentiation of these embryogenic structures was achieved when the tissues were transferred to MS medium devoid of growth regulators or to a medium supplemented with NAA or 2, 4-D. Differentiation of the tissues demonstrated that somatic embryos can be recovered at the rate of 450 embryos per gram of FEC tissues over a period of six weeks. Histodifferentiation of the FEC resulted in malformed cotyledon-stage embryos, which when subjected to maturation, germination and organogenesis using three different strategies did not regenerate into plants. Histological cuttings of the embryogenic tissues confirmed the malformation and absence of meristem. Up to 50mg L⁻¹ of kanamycin is required to arrest embryogenic potential of green cotyledons from somatic embryos. While 50 to 60mg L⁻¹ of paromomycin arrested the proliferation and histodifferentiation of FEC, 5mg L⁻¹ of the antibiotic was sufficient to arrest the proliferation of the cells in liquid medium. Particle bombardment of somatic cotyledons and FEC or FEC-derived embryogenic suspensions using the plasmid pBI426, showed that helium pressures of 900psi and 1,200psi can be used effectively to transform the tissues. Optimal conditions for transient expression of the gus visual marker gene were achieved when tissues were pre-cultured in a medium containing an equimolar mixture of mannitol and sorbitol at 0.2M and bombarded using tungsten particle.

Introduction

In recent times, the increasing economic importance of cassava in most of the northeastern states of Brazil has been brought to focus. This is due, largely to the increasing demand for the crop in the poultry and bakery industries due to high prices of maize and wheat flour, coupled with its traditional use as food and fodder (Machado 2004). Globally, dependence on the crop is expected to increase with a projected 60% increase in production by 2020 (Scott et al. 2000) especially with the crop's potential in the starch industries (Aerni 2006).

The need to increase production to match this demand underscores the corresponding need for more genotypes that are adapted to the prevailing agro-ecological settings in the northeastern states of Brazil. Until recently, tissue culture techniques (meristem culture, axillary proliferation, somatic embryogenesis, organogenesis) and genetic transformation protocols for the cassava genotypes presently used in northeastern Brazil, have not been developed. In order to meet this need, we have made attempts towards developing protocols for the regeneration of cassava plants through somatic embryogenesis and organogenesis of 10 major cassava genotypes. We demonstrate transient expression of reporter genes in a number of these cultivars through Agrobacterium mediated transformation and particle bombardment of embryogenic tissues. Here, we report the progress made in our laboratory on the development of transgenic cassava cultivars with improved tolerance to drought stress.

Cyclic Somatic Embryogenesis

An important prerequisite for the development of genetic transformation system is the availability of morphogenic culture that can easily be used in gene transfer techniques (Taylor et al. 1996, 2004). In cassava, the most efficient way of developing this culture is through somatic embryogenesis. Defined as a process by which a haploid or somatic cell gives rise to a plant while passing through the embryogenic phases similar to zygotic embryos (globular, heart-shaped, torpedo and cotyledon) from non-zygotic cells, without the fusion of male and female gametes (Emons 1994), the process was first reported in cassava by Stamp and Henshaw (1982) from zygotic cotyledons and clonal leaf materials. As in many other plant species, the general strategy for the development of an embryogenic culture in cassava is to induce the formation of somatic embryos from explants on a MS medium (Murashige and Skoog 1962) medium supplemented with auxin from which immature leaf lobes or shoot apices can be used to regenerate plants that are clones of the mature parent material, while preserving all the traits of the given germplasm.

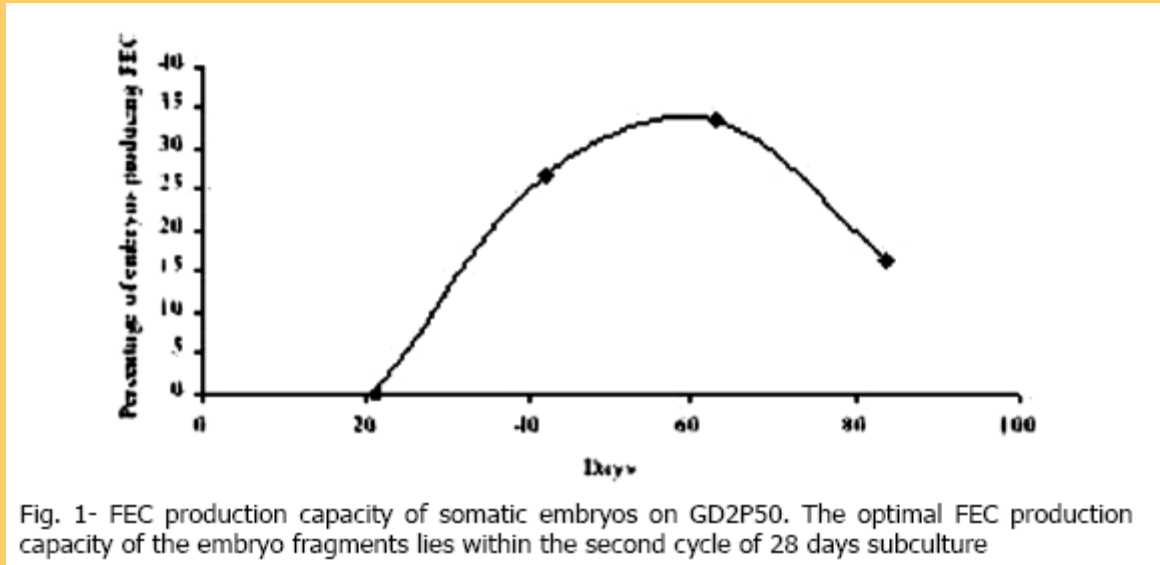
In all the cassava cultivars from northeastern Brazil that were subjected to somatic embryogenesis, primary somatic embryos were induced by culturing shoot apex isolated either from in vitro plants or from mature plants grown under field conditions using cassava induction medium (CIM), which was composed of MS salts (Murashige and Skoog 1962) supplemented with MS vitamins, 2%(w/v) sucrose, 0.5 mg L⁻¹ CuSO₄ and 8 mg L⁻¹ picloram and incubated in the dark. Maturation of embryos was achieved by transferring the primary embryos into MS based medium supplemented with vitamins, 2% sucrose, 0.5mg L⁻¹ CuSO₄ and 0.1mg L⁻¹ BAP (CMM) and incubated in 16 h photoperiod. Through this means, a cyclic system of somatic embryogenesis was established by subjecting cut pieces of cotyledons from mature somatic embryos CIM and sub-cultured every 4 weeks and kept as stock.

Induction and Establishment Friable Embryogenic Callus Lines

We demonstrate transient expression of the reporter gene gus (uidA) in somatic embryos regenerated from transformed cut pieces of somatic cotyledons. However, with the growing concern about the multi-cellular origin of these structures, focus has shifted to a new kind of embryogenic culture based on the production of friable embryogenic callus (FEC). Since its discovery in cassava (Taylor et al. 1996), FEC system has been applied in a number of cassava genotypes of different geographical origins (Raemakers et al. 1993a, b, 1997a, b, Sofi ari et al. 1997, Taylor et al. 2001). The system provides an alternative embryogenic culture in which embryogenic tissues which proliferate in an uncoordinated manner produce friable callus in which the large majority of the cells are totipotent (Taylor et al. 2001). These cells have been identified as ideal targets for use in gene transfer due to their putative unicellular origin (Taylor et al. 1996). The general strategy for obtaining FEC is to culture somatic embryos on GD medium (Gresshoff and Doy 1974) supplemented with 2% sucrose and 33 to 50µM picloram. Following a period of incubation, highly friable and pale-yellowish tissues can be seen emerging from the surface of the embryos. Once induced, maintenance of FEC is relatively simple and suspension cultures can be obtained by transferring a given amount of FEC into SH medium (Schenck and Hilderbrandt 1972) supplemented with 6% sucrose and 50µM picloram. Through this means, gram quantities of the FEC can be obtained and used as target for transgene insertion and prior regeneration of embryos which will subsequently give plants (Taylor et al. 1996, 2001, Schreuder et al. 2001).

We have succeeded in inducing and establishing FEC lines by transferring fragments of somatic embryos from CIM to GD medium with 2% sucrose and 50µM picloram (GD2P50) incubated in a 16 h photoperiod. The initial day of FEC

appearance was recorded for each line obtained and percentage of somatic embryos that produced FEC was counted at the end of every cycle of 3 weeks. From our experiments, the FEC production capacity of the embryo fragments lies within the second cycle of the GD treatment. Lines of FEC from different explants originating from different embryogenic cycles were created in order to evaluate the possible existence of morphological differences among the FEC thus isolated. Scoring the friability of each different line showed that all the lines thrived well even after the date of evaluation and no apparent morphological difference was observed. FEC produced were continuously selected and transferred to fresh GD2P50 for proliferation while what remained of the fragment was further subcultured. Through this means, we evaluated the FEC production capacity of somatic embryos with time. Over 33% of 10 explants of embryo clusters per Petri dish produced FEC over a period of two cycles, giving rise to homogenous and highly friable FEC culture. By the 4th cycle, FEC production of individual embryo fragment decreased (Fig. 1), by which time the entire tissue must have turned into FEC or died up altogether.



Histodifferentiation of Embryogenic Structures

Having thus established a protocol for the production of FEC, we set out to test the ability of the tissues to regenerate in to complete plants through histodifferentiation and germination experiments. Regeneration of the embryogenic tissue into a plant often involves the transfer of the tissue into an MS based medium devoid of growth regulators or supplemented with NAA (Taylor et al. 1996, Schopke et al. 1996, Gonzalez et al. 1998, Taylor et al. 2001). In our work, although cotyledon stage embryos were obtained both on MS medium containing 2% sucrose and 5 μ M NAA (MS2NAA5) and on MS medium with 2% sucrose (MS2), with an average production of over 450 embryos per gram tissue of FEC over a period of 42 days, all the embryos generated were malformed with fused cotyledons (Fig. 2.). Reports exist in which malformed embryos from FEC were obtained (Taylor et al. 2001, Raemakers et al. 2001). It has been recommended that young FEC tissues should be used to regenerate plants as old ones tend to exhibit low regeneration capacity (Raemakers et al. 2001). However, when we subjected 2 months and 1 year old FEC tissues, similar pattern of histodifferentiation with accompanying production of malformed embryos was observed. Embryogenic suspension produced embryos only in media devoid of growth regulators (MS2) and took much longer time (approximately 30 days) to emerge as against the two weeks observed in FEC tissues transferred to MS2NAA5. This was expected, because embryogenic suspension has higher contact with auxin in the liquid medium and would therefore require longer time to eliminate auxin, the removal of which from the medium has been implicated in the triggering of embryogenic competence (Emons 1994). When subjected to serial sieving, the fraction of embryogenic suspension retained by 40 mesh sieve proved to show higher embryo conversion than other fractions.

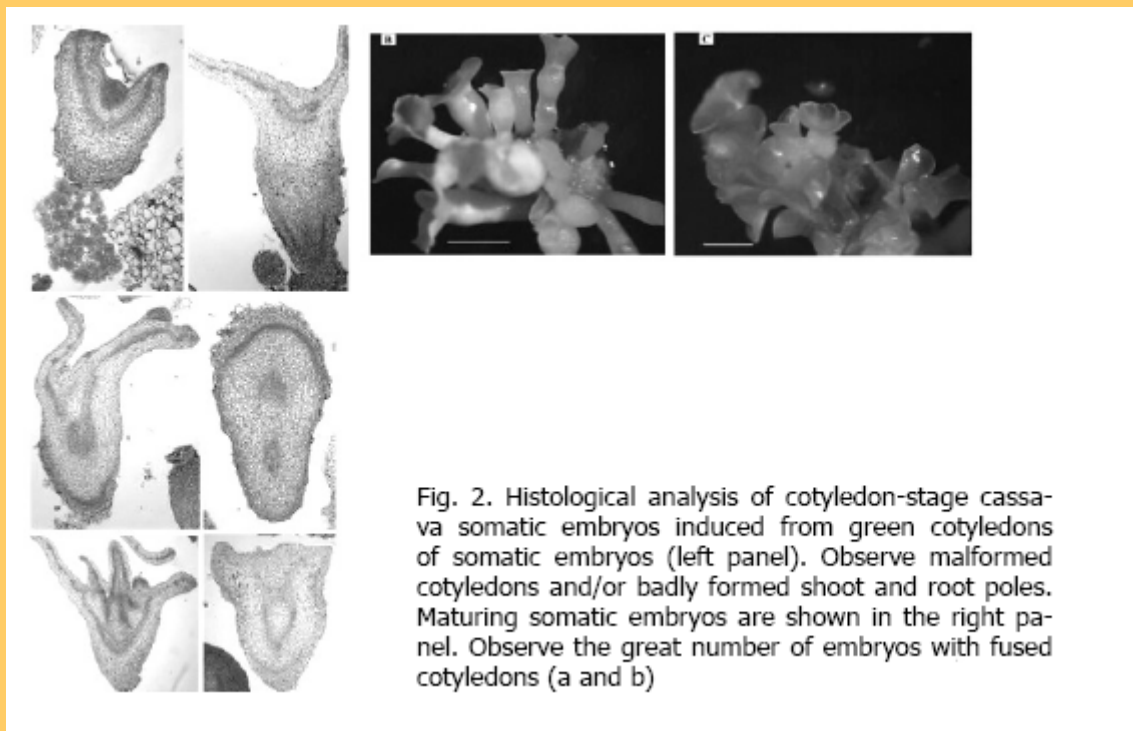
Plant Regeneration

Efforts to germinate the embryos on BAP rich medium met with failure, even when the level of auxin was almost eliminated through desiccation in a medium containing 0.5% activated charcoal (Mathews et al. 1993). We tested three different strategies of regeneration of mature plants from cotyledon-stage embryos emerging from histodifferentiating

embryogenic callus. In the first strategy, individual embryos were cultured in an MS2 medium supplemented with 4.4 μ M BAP, 5.0 μ M BAP and 2.0 μ M BAP. Half of these were kept in the dark while the other half were kept in a 16 h photoperiod. Although none of the embryos regenerated into a plant, active cell division with evidence of foliose structures can be observed in all the treatments. Embryos in the medium containing 4.4 μ M BAP gave relatively more organized and highly enlarged vegetative structures which were erect and sometimes with the emergence of root. Embryos kept in the dark turned pale with no evidence of shoot growth. In the second strategy, the embryos were first inoculated in an MS2 medium containing 0.5% activated charcoal for 1 week, before transfer to the 3 different mediums described above. Similar morphological responses were observed. In the third strategy, cut pieces of green cotyledons emerging from FEC were inoculated in two different organogenesis medium one composed of MS2 with 1 μ M BA and 2.5 μ M IBA (Puonti-Kaerlas et al. 1997) and the other composed of MS2 with 1 μ M BAP, 0.5 μ M IBA, 0.5mg L⁻¹ CuSO₄ (Machado 2004) and incubated in the dark for 20 days. The cotyledons produced callus and sometimes embryo-like structures but never shoot primordium. Currently, efforts seem to be shifting towards the induction of organogenesis directly from FEC (Hankoua et al. 2006). It is however, subject to experimentation, whether the system is reproducible in other cultivars of cassava.

Histological Analysis

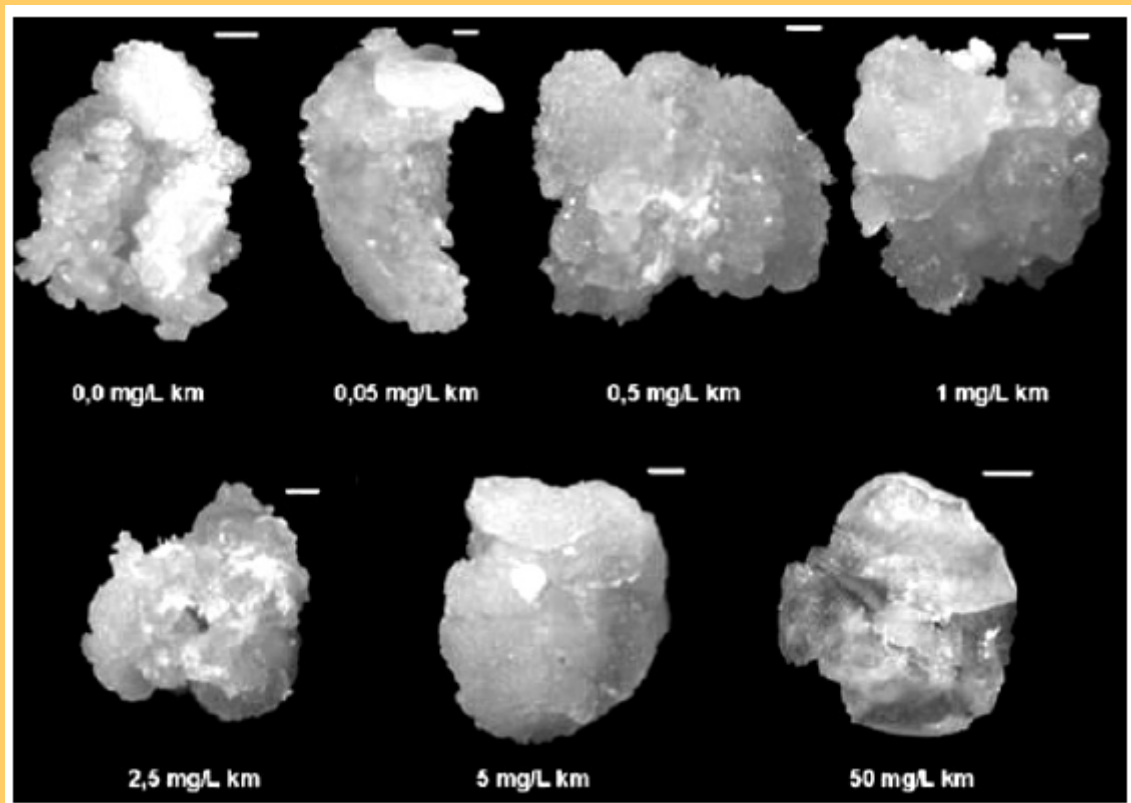
As part of our effort check the “inregenerability” of the embryos, we made an attempt to evaluate the underlying cellular changes that accompany the embryogenic development. Histological cuttings obtained not only confirmed the malformed nature of the embryos, but also indicated the absence of meristem in the apparently bipolar structures obtained (Fig. 2.). A question has arisen as to whether these deformities were as a result of mutation or were induced at certain stage of embryogenic development and elicited by auxin. Our result underscores the need for further investigations with a view to acquiring thorough knowledge of the morphological development, cellular and molecular basis of FEC induction and histodifferentiation.



Since the conversion of globular embryos appears to be the limiting step for the regeneration of plants from FEC (Puonti-Kaerlas 2001), it follows that the success of any regeneration system of FEC must depend on strategies that will take advantage of the underlying competence of these structures. We are inclined to believe that the malformed structures obtained in our regeneration experiments might have been caused by a certain aberration in the expression of morphogenic competence of the cells.

Determination of Phytotoxic Levels of Antibiotics for Selection of Putative Transgenic Tissues

To meet a part of the requirements for the development of transformation systems in cassava cultivars from northeastern Brazil, we subjected both somatic embryos and FEC or embryogenic suspension to a series of killing curve experiments using kanamycin and paromomycin. Increasing the concentration of kanamycin in CIM cultured with cut pieces of cotyledons led to decrease in their embryogenic potential as evidenced by reduction in the frequency of embryogenesis and the number of embryos produced (Fig. 3. upper plate). There was also a dramatic increase in the production of callus with increasing kanamycin during embryo maturation except in the treatment with 50 mg L⁻¹, where the explants died completely. The decrease in somatic embryogenesis and concomitant increase in callus with increasing concentration of kanamycin may be due to hormonal imbalance mediated by cellular degradation of the antibiotic (Lin et al. 1995). Although our investigations show that 50 mg L⁻¹ of kanamycin efficiently selects for putative transgenic embryos, slightly lower concentrations are recommended. At 10 mg L⁻¹, kanamycin inhibited up to 93% of embryos maturation. Callus production was discreet at concentrations below 5 mg L⁻¹ with slight increase through 10mg L⁻¹. At 25mg L⁻¹ and above there was evidence of tissue oxidation (Fig. 3. lower plate). We recommend that 10 mg L⁻¹ of kanamycin is sufficient to select for embryos during maturation.



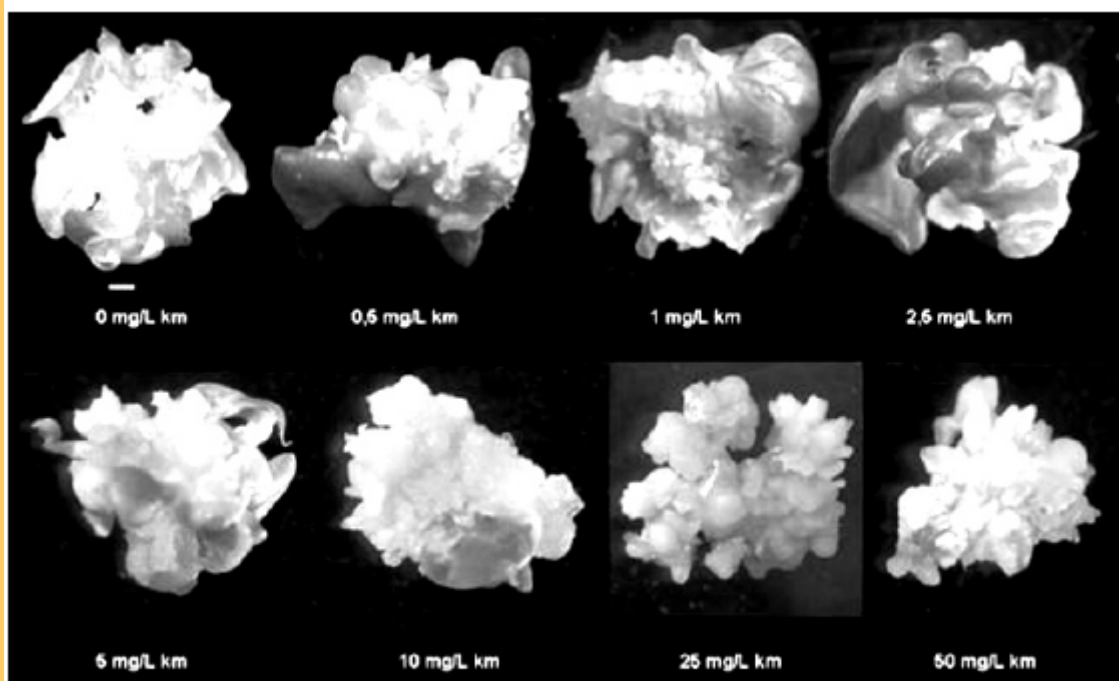
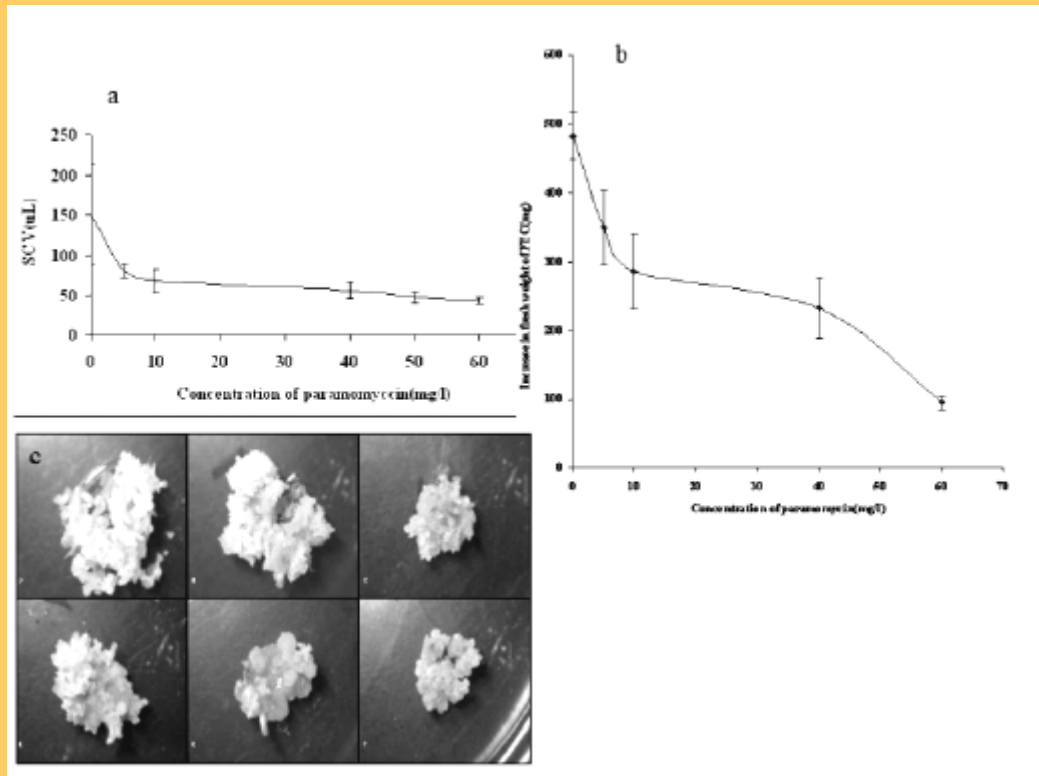


Fig. 3. Effect of kanamycin on somatic embryogenesis demonstrating the production of callus accompanied by decrease in embryogenic potential (upper plate) and arrest of embryo maturation (lower plate)

A killing curve using different concentrations of paromomycin showed that 100mg of FEC proliferates and increases in fresh weight on GD2P50 medium supplemented with up to 45mg L⁻¹ paromomycin. At 60mg L⁻¹, there was an arrest of FEC proliferation and loss of friability (Fig. 4b). This is true for both embryogenic suspension in liquid media and for FEC subjected to histodifferentiation (Fig. 4a and 4c, respectively). While as little as 5mg L⁻¹ of the antibiotic was sufficient to arrest the growth of and kill majority of the cellular suspensions as evidenced by the reduction in the settled cell volume (SCV) and viability test (result not shown), 60mg L⁻¹ promptly killed the cells with accompanying disintegration of the same. A phenomenon observed in both proliferating and histodifferentiating FEC is that, at lower concentration (usually 5mg L⁻¹), there appeared to be a pronounced growth of different types of callus which were often lighter in weight than homogenous FEC (Fig. 4c). It is probable that lower concentrations of paromomycin also tend to interfere with hormonal balance leading to the production of large amount of callus.



Optimization of Parameters for Transient Expression of GUS

Results from *Agrobacterium*-mediated transformation experiments using three different strains indicate that higher co-culture time reduces of embryogenic potential of the explants (results not shown). We are presently screening a collection of *Agrobacterium tumefaciens* strains in order to identify more suitable ones for use in future transformation events. Due to the comparative advantage of genetic transformation via particle bombardment (Altpeter et al. 2005), we used embryogenic tissues to carry out a preliminary evaluation of working parameters in particle bombardment of cassava. The interdependence among different bombardment parameters such as plasmid type, cultivars and pressure has long been established as crucial point in developing a transformation system for any species. In our experiments, the highest frequency of transient expression as well as the number of blue spots on mature somatic embryos was observed when tungsten particle with size $0.5\mu\text{m}$ (M5) was used along with 900psi helium pressure against the combination of M10 and 1,200psi. The explants presented small but well defined blue spots with higher distribution in (Fig. 5a.). The combination of bombardment particles with small size and relatively low pressure has been shown to have a positive effect in generating high frequencies of transient expression in different plant species (Yang et al. 1999, Devi and Sticklen 2002, Tee and Maziah 2005).

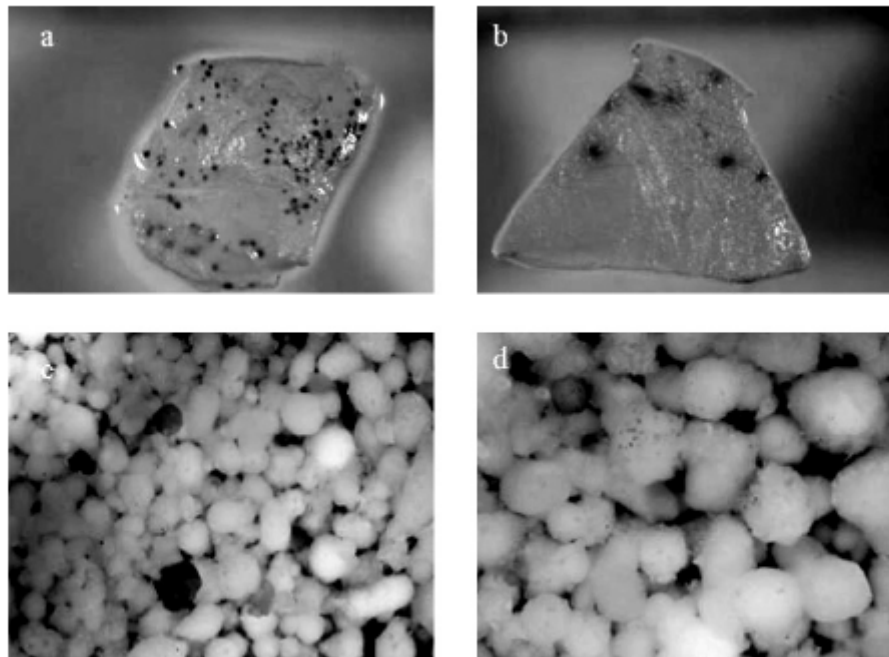


Figure 5. Transient expression of GUS in cotyledons from somatic embryos and FEC 24hrs. Higher expression was observed in the cotyledons with the combination of M5 and 900psi (a) against M10 with 900psi (b). Bombardment of plasmolysed FEC with 1,200psi gave higher expression when M5 (c) particle was used against M10 (d)

Similar experiment was carried out using FEC/embryogenic suspension. It has been demonstrated that plasmolysis treatment of embryogenic tissues with 0.2M mixture of mannitol and sorbitol before and after bombardment are beneficial in particle bombardment of FEC (Schopke et al. 1997). Using a helium pressure of 1,200psi, we studied the effect of plasmolysis and particle size on the transient expression of GUS from bombarded FEC. Histochemical analysis of FEC tissues and embryogenic suspension was carried out 24 h after bombardment. Although the overall frequency of transformation in both treatments seems to be extremely low, the relatively higher level of blue spots observed in the plasmolysed tissues shows that this treatment has a positive effect on the frequency transformation. The optimal condition for transient expression was achieved when tissues were treated with 0.2M mannitol and sorbitol using tungsten particle M5 (Fig. 5a). Following this, large scale experiments were carried out in which, two kinds of embryogenic tissues (FEC that was proliferating in solid medium and embryogenic suspension from liquid medium) were bombarded with pBI426 (Datla et al. 1991) using M5 particle and a pressure of 1,200psi. 24 h after bombardment, $1.6 \pm 0.13924\%$ of intact FEC units gave positive GUS test while $1.142742 \pm 0.242142\%$ embryogenic suspension gave positive GUS test. Selection regime with paromomycin was started one week after bombardment.

Reviewing the progress made in FEC transformation of cassava, Raemakers et al. (2001) highlighted such limitations as the amount of tissue that can be used per bombardment and the number of bombardments necessary to produce desired transgenic plants. Such factors depend on the type and stringency of selection and the characteristics of the traits introduced, which in turn depend on the genotype. These parameters underscore the need to carry out preliminary evaluation of bombardment conditions on any genotype to be transformed.

Selection of Bombarded Embryogenic Structures

Selection of the bombarded tissues using paromomycin was started one week after bombardment. Different reports used paromomycin (Schopke et al. 1996, Gonzalez et al. 1998, Taylor et al. 2001) as a selective agent following the bombardment of embryogenic tissue. Although no embryos emerged from the tissues placed in the histodifferentiation medium (from both types of tissues), when the transient assay was repeated 5 to 6 weeks after bombardment, an average of 3.6% for embryogenic suspension and 3.5% for FEC was observed. It has been reported that the use of paromomycin selection reduces the regenerative potential of the transgenic material (Puonti-Kaerlas 2001). Reasons

such as this are often given in defense of selection systems based on combining antibiotic selection using phosphinotricin and luciferase (Raemakers et al. 1996). Although the use of luciferase may sometimes allow the development of both transformed and non-transformed maturing embryos from bombarded FEC, screening could be used to exclude escapes, while inclusion of the antibiotic during embryo maturation could help in regenerating a number of transgenic lines. However, the use of luciferase detection method may not be practicable despite its non-destructive nature, because it requires access to costly equipment (Puonti-Kaerlas 2001). An alternative system which combines the visual GUS assay with positive selection using mannose seems promising (Zhang and Puonti-Kaerlas 2000), but will only be beneficial if it ensures the elimination of non-transformed tissues without compromising the regenerative potential of the putative transgenic tissues. In summary, the results from our work will form the basis for the transfer of drought resistance and other agronomically important genes in cassava cultivars of northeastern Brazil. Since the low regeneration rates or regeneration of abnormal plants reduces the transformation efficiencies obtained after transformation (Taylor et al. 1996, Raemakers et al. 1997, Schopke et al. 1997, Munyikwa et al. 1998, Zhang et al. 2000) for any transgenic program in cassava to be successful, the target must be the development of a reproducible protocol that will allow for reduction in the length of time required for tissue culture before plant regeneration while ensuring single insertion of the transgene. Efforts to develop new protocols of plant regeneration based on combining the now usual pathway of histodifferentiation and organogenesis (Hankoua et al. 2006) as well as the employment of strategies that will create conditions similar to seed development (Schmidt et al. 2005) seem promising in improving this.

Acknowledgements

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